High-throughput screening of biomolecules using cell-free gene expression systems

Luis E. Contreras-Llano and Cheemeng Tan*

Department of Biomedical Engineering, University of California Davis, Davis, CA, USA

*Corresponding author: E-mail: cmtan@ucdavis.edu

Abstract

The incorporation of cell-free transcription and translation systems into high-throughput screening applications enables the *in situ* and on-demand expression of peptides and proteins. Coupled with modern microfluidic technology, the cell-free methods allow the screening, directed evolution and selection of desired biomolecules in minimal volumes within a short timescale. Cell-free high-throughput screening applications are classified broadly into *in vitro* display and on-chip technologies. In this review, we outline the development of cell-free high-throughput screening methods. We further discuss operating principles and representative applications of each screening method. The cell-free high-throughput screening methods may be advanced by the future development of new cell-free systems, miniaturization approaches, and automation technologies.

Key words: synthetic biology; high-throughput screening; cell-free systems; directed evolution

Introduction

High-throughput screening methods analyze the interactions between a large number of chemical compounds or biomolecules and specific targets in a robust, time efficient and highly reproducible format. This screening technology was initially driven by the increase in the number of compounds and molecules available for testing, and the necessity to accelerate the research and development of new drugs and therapies (1–4). For the last two decades, high-throughput screening assays have advanced significantly based on the development of miniaturized systems (5, 6), more sensitive detection methods (7–9), better data analysis (10) and automation of screening procedures (4, 11). Indeed, high-throughput screening has been used successfully in a wide range of applications such as drug discovery (12), evolution of proteins (13), enzyme engineering (14), as well as screening and discovery of chemical probes (15), small molecules (16, 17) and lipopeptides (18).

Despite the considerable increase of sample processing capacity and quality of the screening assays, the field has relied primarily on the use of *in vivo* (or cell-based) approaches, and

the use of highly purified or chemically synthesized target proteins. *In vivo* approaches exploit the change of cellular phenotype to screen for the presence of specific molecules (Table 1). The *in vivo* approaches are useful when the presence or the activity of the biomolecule of interest can be determined using fluorescence, luminescence, or altered cellular physiology. However, they are not optimal for the screening of binding between two molecules (e.g. identification of small molecule targets of an antibody), or if the assayed molecule is toxic to the host cells. The use of purified or chemically synthesized proteins in high-throughput screening assays reduces interference with other molecules and false positives. Both approaches exhibit certain disadvantages; chemical synthesis is only capable of generating small peptides, and the use of purified proteins requires purification steps that increase the cost and time of the screening procedure.

These limitations have been addressed through the development of high-throughput screening assays that use cell-free transcription and translation systems (Table 1). The cell-free systems synthesize biomolecules encoded on DNA libraries, which are then tested in defined media for an increased

Table 1. Advantages and disadvantages of *in vitro* and *in vivo* methods for the high-throughput evolution, screening and selection of biomolecules

	In vitro (cell-free) approaches	*In vivo* (cell-based) approaches
Principle	The approaches use cell-free transcription–translation systems for the synthesis of biomolecules encoded in genetic materials. The biomolecules are then tested in reconstituted environments	The approaches use live cells for the synthesis and subsequent screening of biomolecules. The biomolecules are screened in natural cellular environments.
Advantages	Toxic or insoluble proteins can be synthesized and screened. Overall experimental time is reduced by omitting cell transformation/transfection and growth processes. Unnatural or isotope-labeled amino acids can be incorporated into synthesized proteins.	The enzymatic activity of a biomolecule can be linked to cellular physiology for rapid screening. Relatively inexpensive due to the use of living cells that can be easily grown and reproduced. The identified biomolecules will work in the target cellular environments.
Disadvantages	Reagents and equipment are expensive. Complex post-translational modification of biomolecules is challenging. The identified biomolecules may not work in desired cellular environments due to misfolding or pleiotropic interactions with cellular components.	Limited to biomolecules that are not toxic to the host cells. Non-specific interactions with cellular components may increase false positive or negative rates. Throughput is low due to the time required to expand cells. Incorporation of non-natural amino acids requires modified host cells.

enzymatic activity through affinity chromatography methods or detection of fluorescence reporters. The use of cell-free systems adds an additional layer of complexity due to the high diversity of molecules present in the analysis solution, the increased possibility of non-specific interactions with cellular components and the myriad ways of controlling protein expression. Despite this complexity, cell-free protein expression systems bring forth several advantages over conventional cell-based approaches, including the capability to express toxic or insoluble proteins (19), the incorporation of unnatural or isotope-labeled amino acids into the peptide chain (20, 21), reduced processing time (22) and reaction volumes (23), as well as the lack of gene-cloning steps (24). These advantages make cell-free systems ideal for high-throughput applications (25, 26).

The techniques that use *in vitro* cell-free transcription/translation systems for the screening, selection and evolution of biomolecules in a high-throughput format can be classified as *in vitro* display and on-chip technologies. Here, we define *in vitro* display technologies as methods that link genotype and phenotype by covalent linkage between proteins, ribosomes, DNA and RNA. After expression, these complexes are directly screened and tested for activity. The linked-genetic sequence is then used in subsequent rounds of enrichment. On the other hand, we define on-chip or microarray technologies as methods that immobilize expressed proteins into treated surfaces without the source RNA or DNA. Several reviews have summarized and analyzed different aspects of these two classes of cell-free high-throughput screening techniques (8, 27–29). In contrast, we aim to underline the development of the central techniques that broaden the adaptation of cell-free expression systems for the high-throughput screening, selection and evolution of biomolecules. We focus our efforts on explaining how each one of the technologies was developed in a connected timeline and modified to tackle problems in the preceding techniques. We also review the techniques with increasing complexity of compartmentalization: (i) without physical compartmentalization; (ii) 2D microarrays in which the biomolecules are attached to a treated surface; (iii) 3D micrometer scale compartments. Furthermore, we summarize all the techniques regarding their main advantages and disadvantages (Figure 1). We also emphasize the chronological development of these technologies

(Figure 2) to show how the advancement of *in vitro* display and on-chip technologies has been enabled by discoveries in other scientific areas.

Screening of biomolecules in a single pot without physical compartmentalization

Polysome and ribosome display

Polysome and ribosome display are the first fully *in vitro* approaches developed for the selection and evolution of small peptides and native folded proteins respectively (30, 34) (Figure 3A). Ribosome display adapted the pioneering method used by *in vitro* polysome display for the screening of a large library of decapeptides displayed on polysomes using affinity selection towards an immobilized antibody (34). The method intentionally omits the stop codon so that a peptide expressed using an *E. coli* S30 system remains attached to the ribosome while ensuring that the peptide folds properly. In this study, the authors synthesized single-chain fragments of an antibody with disulfide bonds and screened the antibody fragments using an antigen. Through this proof of concept, the authors showed that it was possible to carry out phenotypic selection (i.e. ligand binding) using completely native proteins that were synthesized *in vitro*. The method has two main advantages over the cell-based selection and screening technologies. First, the diversity of the library is not limited by the transformation efficiency of host cells. Instead, the quality of the library is controlled only by the number of ribosomes and mRNA. Second, a new round of diversification can be introduced to the library after the end of each selection round, hence overcoming the need to transform cells after each diversification step in the cell-based screening methods (35). The ribosome display method has been applied to select peptides with increased activity towards prostate-specific antigen (36), streptavidin (STA) binding peptides that confer an increased affinity to bovine heart fatty acid-binding protein (FABP) (37) and picloram-specific variable fragments of heavy chain antibodies (38). Polysome and ribosome display are the first methods that demonstrate the *in vitro* linkage between genotype (mRNA) and phenotype (small peptides and proteins respectively). However, their main disadvantage is the difficulty in displaying certain peptides due to their
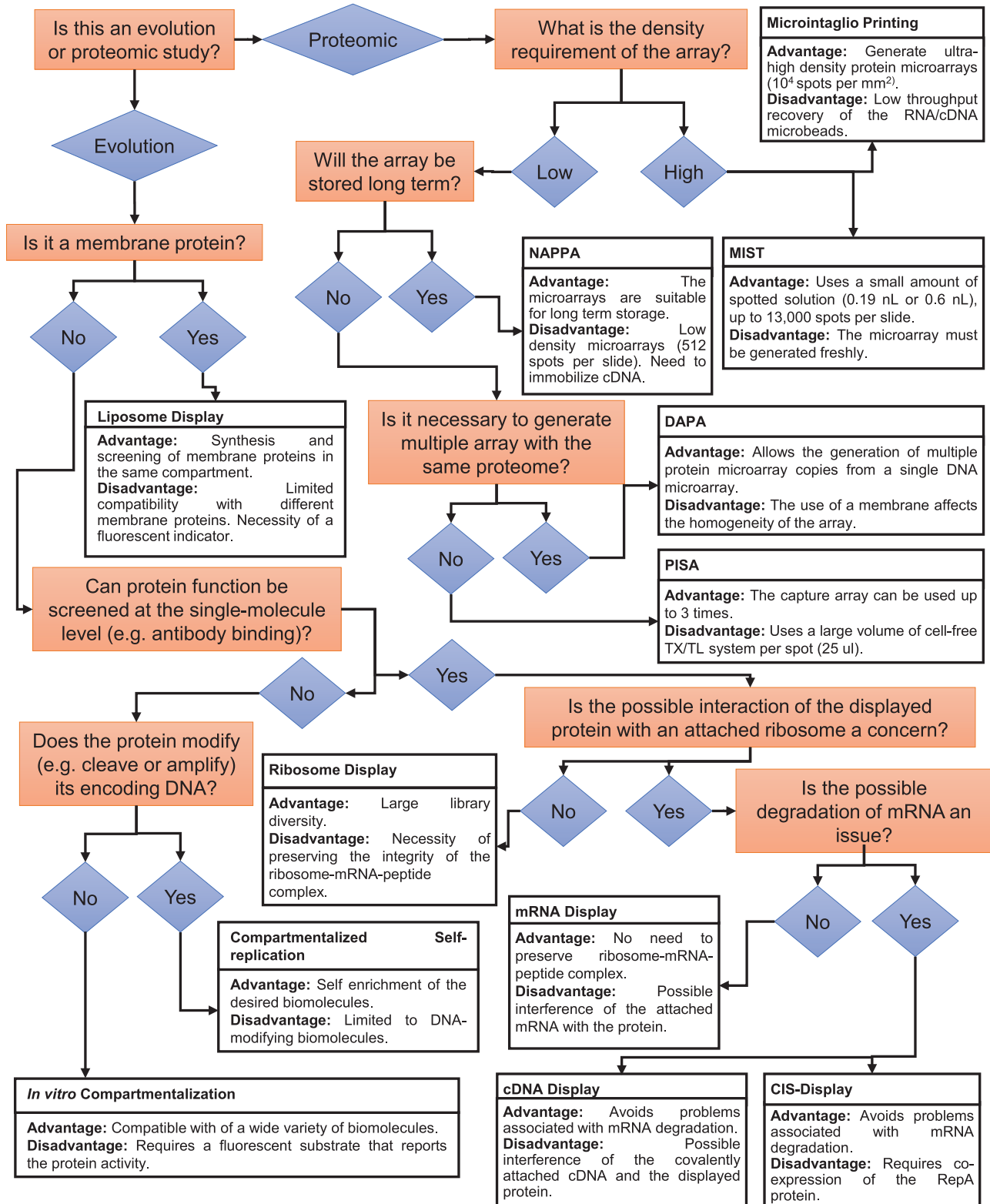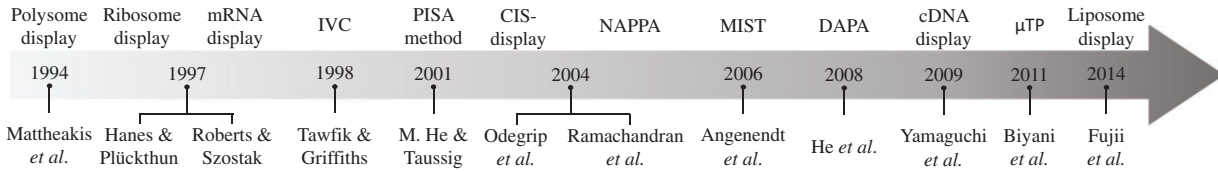
Figure 1. A summary of the techniques that incorporate cell-free protein expression systems for the high-throughput screening, selection and evolution of biomolecules. This flowchart outlines the choice of a technique based on the experimental requirements as well as provide a brief description of the advantage and disadvantage of each technique.

Figure 2. Chronology of the techniques used for the high-throughput screening of biomolecules using cell-free gene expression systems. The timeline shows the authors and their invented techniques that use cell-free transcription/translation systems for the high-throughput screening of biomolecules.
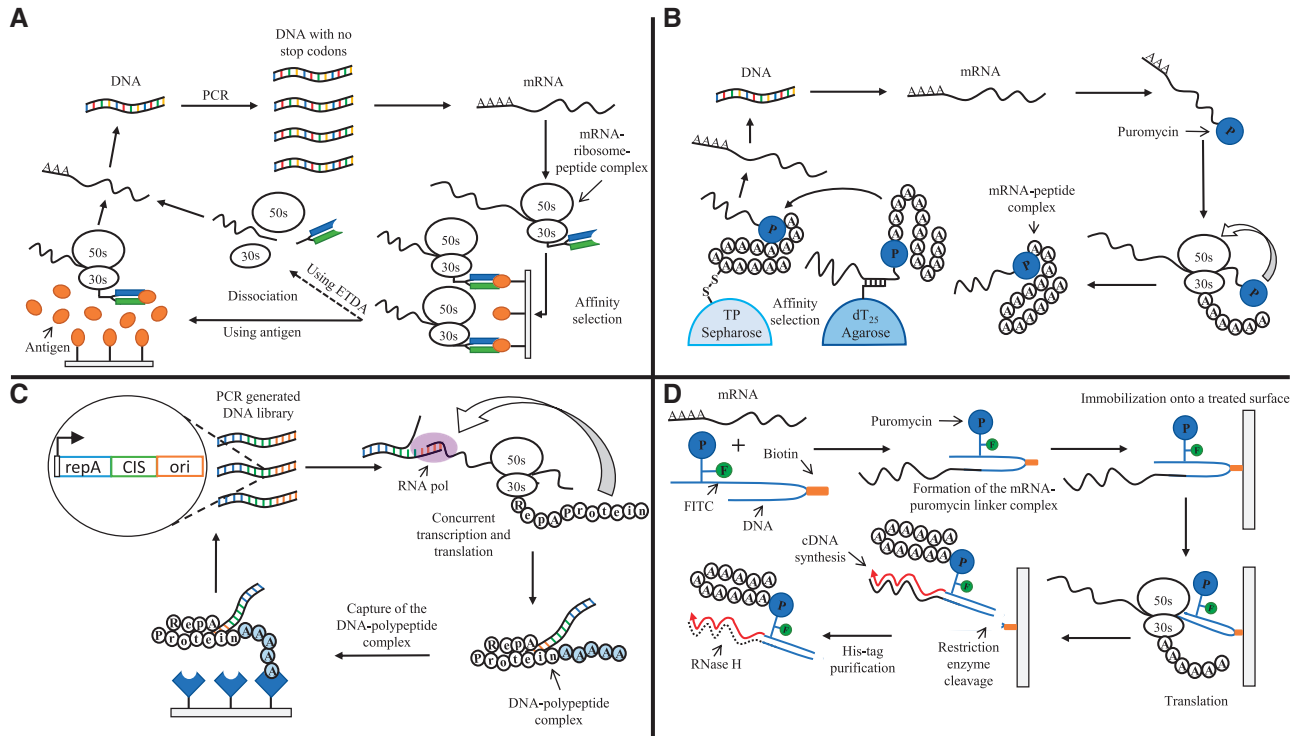
Figure 3. Techniques for the screening of biomolecules without physical compartmentalization. (**A**) Graphic representation of *in vitro* ribosome display, adapted from (30). This technique uses polymerase chain reaction (PCR) to amplify DNA without stop codons, allowing the synthesized mRNA to stay attached to the ribosome, while the generated peptide folds properly. Then, the mRNA-ribosome-peptide complexes are affinity selected for subsequent reverse transcription into cDNA. (**B**) Graphic representation of *in vitro* selection (mRNA display), adapted from (31). mRNA display uses mRNA molecules linked to puromycin (P) to generate a mRNA-peptide complex. The resulting molecule is then selected by affinity chromatography and reverse transcribed to generate DNA. (**C**) Graphic representation of the CIS-display technology, adapted from (32). This method uses a property of the RepA protein that transiently interacts with the CIS element. A peptide is co-expressed with the RepA protein. When the RepA protein interacts with the CIS element found in the DNA, the interaction produces a linkage between the DNA and the polypeptide. The resulting molecule can be captured using an immobilized target and PCR amplified for the next round of selection. (**D**) Graphic representation of the cDNA display method using a puromycin linker DNA, adapted from (33). mRNA and the puromycin linker are ligated using T4 ligase. The product of this reaction is then immobilized onto a treated surface using a biotin adapter attached to the DNA linker. The immobilized mRNA is then translated using a cell-free expression system. The resulting protein stays attached to the ribosome, and it fuses to the linker through the puromycin molecule. The mRNA is reverse transcribed, and the immobilized complex is released using a restriction enzyme. After affinity chromatography purification, the mRNA is digested with RNase to confirm the presence of the cDNA-peptide fusion.

unpredictable interactions with the attached ribosome (39). A new display technology that addresses this issue was developed swiftly.

mRNA display

Shortly after the development of the ribosome display technology, the mRNA display method is developed for the *in vitro* selection and directed evolution of proteins (31) (Figure 3B). This methodology uses synthetic mRNAs that carry puromycin at their 3′ end. This approach avoids the necessity of preserving the integrity of the ribosome-mRNA-peptide complex required in the ribosome display methodology. Instead, it uses a mRNA molecule that serves as both a messenger and an adapter. Puromycin is attached to the 3′ end of the synthetic mRNA, and it is an antibiotic that mimics the aminoacyl end of tRNA. Using this characteristic, the protein of interest is expressed using a reticulocyte lysate. The expressed protein then inhibits the translation by entering the ribosomal A site yielding a peptidyl-puromycin fusion molecule as a result of the peptidyl transferase activity of the ribosome. Taking advantage of this reaction, this method generates a stable mRNA-peptide fusion molecule that can be then purified using affinity chromatography followed by immunoprecipitation. The DNA sequence can also be recovered by reverse transcribing the mRNA.

To demonstrate the utility of this technique, a fusion of the long myc template (LP154) and its synthetic mRNA was

enriched from a pool of random sequence templates (LP160). Comparing the change of the ratio between pool/myc, the authors determined that the myc sequence was enriched 20- to 40-fold. The mRNA display has been used in the evolution of antibody mimics (40) and single-chain antibodies (41), the selection of high-affinity aptamers (42) and the identification of drug receptors (43). We point the readers to a few comprehensive reviews about this technique (44–46).

CIS-display

The CIS-display method (32) takes advantage of the cis-activity inherent of the RepA protein (a DNA replication initiator protein) that binds to the template DNA from which it has been expressed (Figure 3C). The template DNA contains a CIS element that composes of a Rho-dependent transcriptional terminator, which stalls the RNA polymerase. The stalling of RNA polymerase allows the newly synthesized RepA protein to bind to the CIS element that switches RepA to the adjacent *ori* site. To create the DNA library, DNA fragments of random DNA sequence are ligated with a DNA fragment that encodes RepA and carries the CIS element and an *ori* site. These DNA fragments are used as templates in the *E. coli* S30 expression system that generates protein-DNA complexes due to the stable association of each protein with the DNA that encodes.

CIS-display technology is intended to overcome the size limitations of *in vitro* selection systems based on emulsion encapsulation that generates DNA mutant libraries of 10^9 to 10^{10} per ml. The authors demonstrated the utility of the CIS-display method by creating a library encoding $>10^{12}$ random 18-mer peptides and showed >1000-fold enrichment of peptides in each round of affinity selection against a relevant target. The selection of peptide ligands was performed using two well-characterized antibodies (anti-P53 DO1 and anti-FLAG M2) and lysozyme. This technique has also been used select a high-affinity binder to the extracellular region of human vascular endothelial growth factor receptor isoform 2 (VEGFR-2) (47) and has been coupled to next-generation sequencing and bioinformatics to facilitate the design of peptides with a potential therapeutic target (48).

cDNA display

The cDNA display method (Figure 3D) is an improvement of the mRNA display technology, originally developed as an approach for the screening of disulfide-rich peptides (33). The method uses a cell-free translation extract, a puromycin-linker DNA containing a ligation site, a biotin site that also serves as the reverse transcription primer site and a restriction enzyme recognition site. These features allow several crucial processes to happen: (i) the rapid ligation of mRNA and the linker, (ii) a biotin/STA-based purification step and (iii) cDNA synthesis by reverse transcription. Taken together, these characteristics prevent the degradation of mRNA, reduce the time employed in the procedure and allow the conversion of mRNA display (mRNA-protein fusion) to cDNA display (cDNA-protein fusion).

As a proof of concept, the authors screened several test proteins (i.e. immunoglobulin G, POU-specific DNA-binding domain of Oct-1 and anti-FLAG antibody) for specific disulfide patterns. This technology has been successfully applied to the screening of Growth Hormone Secretagogue Receptor-binding peptide (49). Since its introduction, several studies have improved the method by making it more robust, practical and convenient through the design of new puromycin-linkers (50, 51), a pull-down method that uses biotinylated bait protein (52) and a puromycin-linker containing 3-cyanovinylcarbazole nucleoside (cnvK) (53).

Screening of proteins on 2D microarrays

Cell-free systems can be used to synthesize proteins using genomic sequences for high-throughput proteomic studies (54). One major class of methods synthesize either each protein one-by-one (19, 55) or the entire proteome in a single reaction (56). Another class of methods synthesizes defined proteome on microarrays for repeated high-throughput assays. In this review, we focus on the later microarray-based methods.

Protein in situ array method

A protein microarray or protein chip is a solid surface (that typically consists of chemically treated glass) on which different proteins are immobilized in a high-density format (57). Protein *in situ* array (PISA) method is the first protein microarray technology incorporating a cell-free expression system for protein synthesis based on DNA as a template (58) (Figure 4A). This method is renamed DiscernArray™ in a subsequent study in which the authors used polymerase chain reaction (PCR) to generate genes or gene fragments as templates to produce histidine-tagged proteins using a rabbit reticulocyte transcription/translation system (62). Irrespective of the expression systems, the synthesized proteins are attached to a Ni-Nitrilotriacetic acid coated surface shortly after their expression to generate the protein array. In the first description of PISA method, it was used to produce arrays of single-chain antibodies and luciferase in reaction volumes of 25 μl. In a subsequent study, the volume of cell-free protein expression was decreased to 100 nl using piezoelectric dispensers and by incorporating a commercially available *Escherichia coli in vitro* transcription translation system. The authors of this study demonstrated high-throughput screening of the enzyme β-galactosidase in nanowells and generated proteins in sufficient quantity for detection by confocal laser scanning (63).

Nucleic acid programmable protein array

The next advancement of the protein array technology is described in a study that introduces nucleic acid programmable protein array (NAPPA) as a high-throughput methodology to study protein function through the expression of cDNA clone collections using mammalian reticulocyte lysate (64) (Figure 4B). High-density peptide/protein chips are fabricated by capturing nascent polypeptides during translation onto a solid surface (65). This peptide-capture mechanism takes advantage of the delay in the release of a ribosome that encounters a double-stranded RNA or RNA–DNA hybrid region at the end of the mRNA. This phenomenon provides both the time and the required physical conditions for a puromycin, grafted on an oligo, to enter the A site of the to-be-released ribosome, resulting in synthesized peptides or proteins that are immobilized on a solid glass surface. The authors demonstrated the utility of NAPPA by mapping the interactions among 29 proteins involved in the human DNA replication complex. In a further improvement of the original NAPPA method, the authors demonstrated the expression of over a thousand different human proteins including membrane proteins and proteins over 100 kDa on a microarray surface (66). NAPPA has also been used in the detection of antibodies that bind to tumor antigens in breast cancer (67), the profiling of serological autoantibodies of type 1 diabetes (68) and
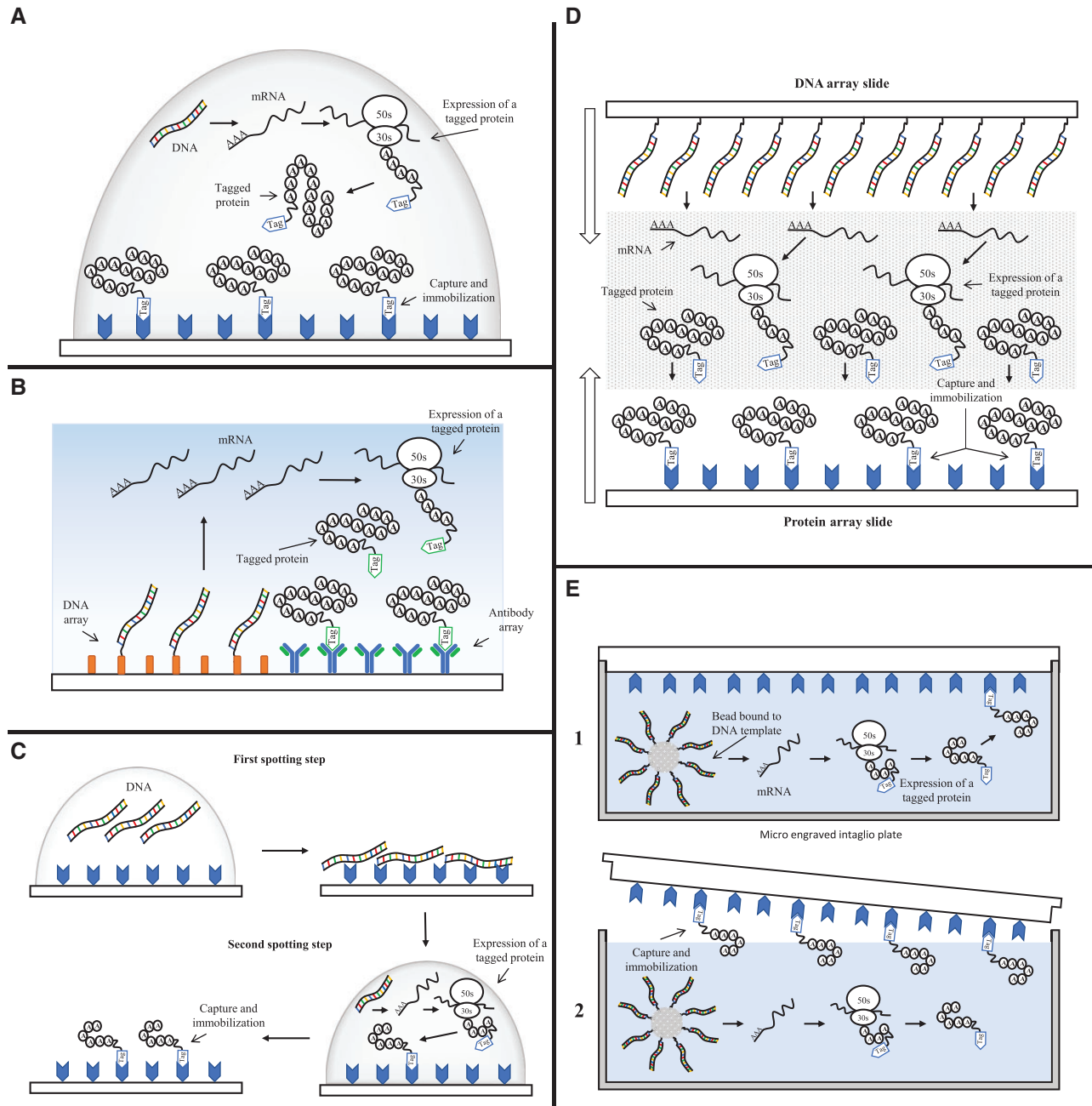
Figure 4. Techniques for the generation of 2D protein microarrays. (**A**) Graphic representation of 'Protein *in situ* array' (PISA), adapted from (59). A droplet of cell-free transcription/translation system and DNA is spotted over a surface modified with a tag-capture agent. After the expression of a tagged protein, it is captured and immobilized on the surface. (**B**) Graphic representation of 'Nucleic acid programmable protein array' (NAPPA), adapted from (59). In this technique, DNA and an antibody that is used as a capture agent are first arrayed onto a surface and then incubated with a transcription/translation system. The proteins expressed using the arrayed DNA as a template are captured promptly by the arrayed antibodies. (**C**) Graphic representation of 'Multiple spotting technique' (MIST), adapted from (29). MIST technique uses two sequential spotting steps for the deposition of a DNA template and a cell-free transcription/translation system. After the second spotting step, the protein is expressed and then captured onto the protein array. (**D**) Graphic representation of 'DNA array to protein array' (DAPA), adapted from (60). A slide with a protein array is brought in contact with another slide with a DNA array and a permeable membrane carrying a cell-free expression system. This configuration allows the protein produced from the DNA array to permeate and bind to the slide with the protein array. (**E**) Graphic representation of microintaglio printing (μP) technique, adapted from (61). This technique uses micro-wells generated by photolithography and a bead bound by a single-copy amplified DNA template. The micro-well is filled with a cell-free transcription/translation system and then sealed with a treated glass surface. After protein expression, the protein is captured and immobilized onto the glass surface, generating a protein array.

the profiling of circulating and synovial antibodies of juvenile arthritis patients (69). We refer the reader to other review papers that discuss the applications of the technique in details (70, 71).

Multiple spotting technique

A couple of years after the development of the NAPPA method, a technique called multiple spotting technique (MIST) emerged

(Figure 4C). The authors applied a multiple spotting technique (72) for the detection of the fluorescent signal and protein expression in nanoliter volume (63). Later, they used this methodology to develop *in situ* synthesized protein arrays (73). MIST uses two consecutive spotting steps on an activated glass slide: the first one is used for the deposition of a DNA template and the second one transfers a rapid translation system (RTS) 100 from *E. coli* on the top of the first spot. After the second spotting step, the slide is incubated in a humid hybridization chamber for rehydration. The rehydration creates separated reaction entities that allow the start of protein expression. When this process has finished, the proteins are detected using specific antibodies. The authors used wild-type green fluorescent proteins (GFP) to demonstrate the creation of microarrays composed of up to 13 000 spots per slide. In addition, they demonstrated that either plasmids or unpurified PCR products could be used as a template. Finally, they synthesized protein arrays using a library of 384 cDNA from human fetal brain cells (73). This technique has also been used in the analysis and characterization of soluble monoclonal antibody fragments (74).

DNA array to protein array

The DNA array to protein array or DAPA is developed for the generation of multiple arrays from an immobilized DNA template (60) (Figure 4D). This approach allows the printing of at least 20 protein arrays from a single DNA template array. The DAPA method uses an array of DNA fragments covalently immobilized to a slide that acts as a template for the synthesis of proteins. This template slide is placed face-to-face with a second slide functionalized with a tag-capturing reagent. The protein expression is carried out using *E. coli* lysate as a cell-free transcription/translation system that is incorporated in a permeable membrane that is sandwiched between the slides that have the DNA array and the tag-capturing array. The proteins once expressed become rapidly immobilized on the capture slide, creating a protein array that can be duplicated several times. DAPA has been extended to print arrays of 116 different proteins (75). It has also been optimized using different array support coatings, which increase its sensitivity towards the printed proteins (76).

Improvement of protein arrays

Some methods have focused on the improvement of the density achieved in protein arrays. Microintaglio printing (μTP, Figure 4E), allows the parallel spotter-free printing of *in situ* synthesized proteins for the creation of high-density protein microarrays directly from mRNAs (77) and later from DNA microbeads (61). The μTP approach uses a micro-engraved intaglio plate fabricated by photolithography and replica molding that generates an array of uniformly 5-μm diameter microchambers at a density of 10^4 per mm². This micromold plate can be filled with rabbit reticulocyte lysate used to synthesize tagged proteins, which thereafter are captured by a treated glass surface. This approach was further improved using poly(dimethylsiloxane) (PDMS) for the creation of a soft lithography-based microintaglio printing method to generate high-density protein arrays (78). A year later, temperature controlled microintaglio printing (TC-μTP) was developed and used to generate RNA microarrays using DNA-immobilized magnetic beads (79). The second and very similar method that aimed to improve the density of protein arrays uses on-chip micro compartmentalization of protein synthesis in arrayed micrometer scale chambers from confined DNA template molecules (80). The authors compartmentalized single genes and using *E. coli* lysate they expressed different fluorescent proteins in microchambers of 7 μm in diameter and 5 μm in depth with a volume close to 200 fl. Similarly, they achieved a density of 10^4 spots per mm².

Protein arrays have been improved using light-directed localization of genes and protein traps (antibodies specific to a peptide tag) through a novel chimera molecule that serves as a surface coating agent (N_ω-Nvoc-amine-N_α-[3-(triethoxysilyl)-propyl]-carboxamide-polyethylene glycol or 'Daisy') (81). The chimera molecule allows the immobilization of dsDNA molecules with a length of thousands of base pairs into a 'Daisy-coated' silicon dioxide surface. The DNA is then transcribed using T7 or sp6 RNA polymerases and translated using a commercial wheat germ-based protein expression system. Using the DNA array, they demonstrated a cell-free gene circuit with a two-stage cascade. This technique was further used to carry out local gene activation using a dual-DNA brush digestion–ligation cascade (82). This digestion-cascade leads to the swapping and rearrangement of DNA resulting in a DNA brush that encodes the complete sequence of GFP under a promoter suitable for expression using an *E. coli* transcription/translation system. The same coating technique was also used in the development of a new method for the parallel synthesis, assembly and imaging of a protein at the nanoscale (83). This approach was used to demonstrate efficient self-assembly of protein nanotubes using GFP, gp 18 (the tail sheath forming a protein of phage T4), STA-conjugated DNA and RecA proteins.
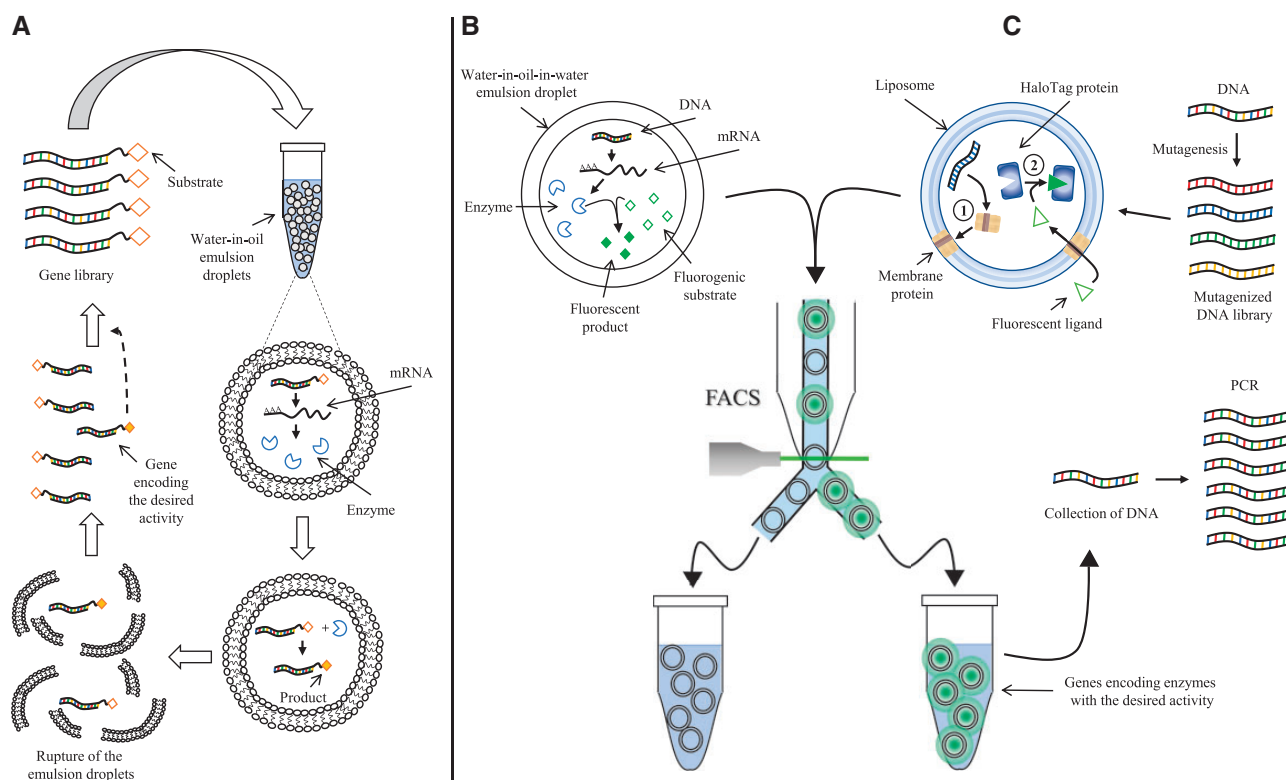
Screening of biomolecules in 3D micrometer scale compartments

In vitro compartmentalization

In vitro compartmentalization (IVC) uses an aqueous compartment containing a transcription/translation or a transcription-only system to enclose and link both the phenotype (protein or peptide of interest) and the genotype (coding DNA). 'Genescis', or gene selection in a compartmentalized *in vitro* system is the first technique to demonstrate the use of compartmentalization for high-throughput screening of biomolecules (84). They compartmentalized an *E. coli* S30 transcription/translation system inside water-in-oil emulsions and used it to express catalytic proteins that were selected based on product formation (Figure 5A). The authors screened for DNA-methylation activity by enriching a methyltransferase (HaeIII) from a 10^7-fold excess of genes encoding different variants of the enzyme. This essay took advantage of the resistance of the product (methylated DNA) to restriction digestion, allowing only the enrichment of the genes that encode HaeIII methyltransferase.

A year later, 'STABLE' or STA-biotin linkage in emulsions is introduced. This method is the first to apply *in vitro* protein selection based on physical association (87). The method is intended for the evolution of proteins and for the selection of cDNA libraries. STABLE uses a water-in-oil compartmentalized *E. coli* S30 transcription/translation system to synthesize STA-fused polypeptides that are attached to their coding DNAs through a biotin label. The resulting protein-DNA fusion molecules are then recovered from the emulsions and subjected to affinity selection with Ni-agarose resin. To test this approach, the authors assessed the efficiency of enrichment of STA-His fusion molecules synthesized from a mixture of STA-His DNA and STA-Random DNA as a model experiment. The results showed a 10-fold enrichment of STA-His relative to

Figure 5. Techniques for the screening of biomolecules in 3D micrometer scale compartments. (**A**) Graphic representation of 'Gene selection by compartmentalization', the first reported technique involving IVC, adapted from (84). A gene library is compartmentalized inside droplets of water-in-oil emulsions. The compartmentalized genes are then transcribed and translated. The synthesized enzymes modify a product linked to the gene inside its compartment. After stopping the reaction, the emulsions are broken, and the genes linked to the product are selectively enriched and can be used for a new round of selection. (**B**) Graphic representation of 'Selection by IVC in water-in-oil-in-water emulsions', adapted from (85). Single genes are compartmentalized in droplets of water-in-oil emulsions and translated in the presence of a fluorogenic substrate. The expressed enzyme converts the substrate into a fluorescent product. The droplets are subsequently isolated using FACS. (**C**) Graphic representation of the liposome display method, adapted from (86). A mutagenized gene library is compartmentalized inside liposomes. Membrane proteins are expressed based on the gene library using a PURE transcription/translation system (see 1 in the figure). The activity of the membrane protein is detected using a fluorescent ligand that is incorporated inside the liposome (see 2 in the figure), and subsequently selected by FACS.

STA-random after a single round of selection. However, the efficacy of the protein–DNA fusion formation was estimated at 1% of the total DNA.

A further study presents a variant of the STABLE system called DNA display (88). The main differences of this method to the original STABLE are the use of a wheat germ transcription/translation system (instead of the *E. coli* S30) to express cDNAs and the incorporation of an antibody as immobilized bait for the recovery of the DNA-peptide conjugates. This approach was used to generate a library of 10^9–10^{10} decapeptides from which 21 were selected using a monoclonal antibody anti-FLAG M2. The efficacy of the formation of protein–DNA fusion increased to >95% due to the use of the wheat germ transcription/translation system.

A method that uses only a polymerase that replicates its own genome, called compartmentalized self-replication (CSR), is developed for the directed evolution of enzymes or auxiliary factors involved in DNA replication (89). Here, the authors use bacterial cells (*E. coli*) as delivery vehicles for a thermostable polymerase and its coding DNA. The resulting product is then cloned again in *E. coli* cells, and the process is repeated, yielding polymerase variants with increased thermostability. As a proof of principle, the authors used three rounds of CSR to evolve a polymerase with 11-fold higher thermostability and 130-times more resistance to the inhibitor heparin.

Multiple variations of IVC have been developed. For instance, the microbead display method (90) uses microbeads to

link proteins to their coding DNA, which can be selected using fluorescence-activated cell sorting (FACS). The authors demonstrated a 10^6-fold enrichment of the gene encoding hemagglutinin after two rounds of selection. This same approach has also been used for the selection of specific ligase ribozymes that act in *trans* (91). A subsequent study describes the use of FACS for the selection and screening of double emulsion water-in-oil droplets containing a fluorescent marker or a fluorogenic substrate (Figure 5B). This approach is used for the enrichment of genes that are trapped together with a fluorescent marker (FITC-BSA) (85). A different variant of IVC called 'Selective gene amplification' (SGA) uses product-specific antibodies for the selection of enzymes and ribozymes (92). SNAP-display is an alternative method that covalently links proteins to their coding DNAs using a SNAP-tag (93).

IVC has been used for the directed evolution, selection and screening of a number of biomolecules. In Table 2, we summarize the applications of IVC found in the literature.

Liposome display

The previous *in vitro* display methodologies are useful for the evolution, screening and selection of soluble proteins and peptides. However, these techniques are not applicable to proteins that require integration in lipid bilayer membranes. To address the challenge, liposome display is designed for the evolution

Table 2. Biomolecules evolved, selected and screened using IVC

	Biomolecule	References
Directed evolution	Ebg β-galactosidases	(94)
	p53 variants	(95)
	β-Glucosidases	(96)
	Cellulases	(97)
	Hexose oxidases	(98)
	Glucose oxidases	(99)
	[FeFe] hydrogenases	(100)
	Proteases	(101)
	Hydrolases	(102)
	Cutinases	(103)
	Arylsulfatases	(104)
	Anti-HER2 DARPins	(105)
		(106)
	Ligase ribozymes	(107)
	Meganucleases	(108)
	Ligases	(109)
Screening and selection	Zinc finger proteins	(110)
	DNA-nuclease inhibitors	(111)
	Ribozymes	(112)
	RNA polymerases	(113)
	Restriction endonucleases	(114)
	RNA-binding proteins	(115)
	Streptavidin variants	(116)
	Bacteriophage λ integrases	(117)
	Hydrogen peroxide generating enzymes	(118)
	T7 promoter variants	(119)
	Esterases	(120)

and selection of membrane proteins (86) (Figure 5C). In this approach, membrane proteins of interest are displayed on the surface of liposome membranes after being translated from single DNA molecules using encapsulated PURE cell-free translation system (121). The PURE system is selected for this approach due to its lack of membrane proteins that may interfere with the activity of the membrane protein of interest. After expression and display, the membrane proteins embedded in the liposomes fold into their functional forms, and the activity of these proteins can then be quantified with a fluorescent indicator. This methodology uses FACS to select the liposomes that display highly functional proteins correlated with a higher fluorescence emission. The sorted liposomes are then lysed to obtain the DNA fragments encoding the highly functional membrane proteins. The authors used this method for the evolution of pore-forming activity of the protein α-hemolysin from *Staphylococcus aureus*. After 20 rounds of selection using liposome display, they obtained a mutant version with 30-fold higher pore-forming activity than the wild-type. A recent improvement of this methodology incorporates the Sec translocon into the liposome display technique to increase the membrane integration efficiency and to expand the range of membrane proteins that can be inserted using this technology (122). Liposome display has been used for the *in vitro* evolution of a mutant pyrrolysyl-tRNA synthetase with improved N-benzyloxycarbonyl-L-lysine incorporation activity (123), and for the evolution of alpha-hemolysin with increased nano-pore activity (124).

Conclusions and visions

We review the high-throughput screening, selection and evolution of biomolecules that use cell-free transcription/translation systems, with special emphasis on how these techniques have been developed, improved and applied. The most recent *in vitro* display and microarray approaches have focused on increasing the range of applications and the screening of more complex biomolecules, such as membrane proteins and proteins that require multiple disulfide bonds. High-throughput screening of biomolecules using cell-free transcription and translation systems may be improved based on advances in several foundational technologies, including the development of better data analysis tools, the creation of new and improved cell-free protein expression systems (125), the advancement in automation of the screening processes (23) and further miniaturization to single molecule level that can still yield a detectable signal.

The vast majority of high-throughput cell-free screening methods strongly relies on the use of FACS or confocal optical instruments for the detection of the biomolecules of interest. However, the use of such equipment in high-throughput screening assays represents a critical bottleneck because they select a few positive hits among a large number of negative results. The use of specialized equipment also slows down the widespread adaptation of these technologies in laboratories with no or very limited access to the equipment. The challenges, if overcome, will speed up the screening procedure and allow for the exploration of larger candidate library in a shorter time than current screening technology. We anticipate the surge of innovative approaches in the near future that will surpass the restriction imposed by screening on 2D surfaces. These new approaches will perform ultra-high-throughput screening assays that exhibit novel characteristics such as autonomous selection, self-amplification of the DNA encoding the biomolecule of interest and a throughput limited only by the size of the library instead of the choice of screening assay.

References

1. Sundberg,S.A. (2000) High-throughput and ultra-high-throughput screening: solution- and cell-based approaches. *Curr. Opin. Biotechnol.*, 11, 47–53.
2. Pereira,D.A. and Williams,J.A. (2007) Origin and evolution of high throughput screening. *Br. J. Pharmacol.*, 152, 53–61.
3. Mayr,L.M. and Fuerst,P. (2008) The future of high-throughput screening. *J. Biomol. Screen.*, 13, 443–448.
4. —— and Bojanic,D. (2009) Novel trends in high-throughput screening. *Curr. Opin. Pharmacol.*, 9, 580–588.
5. Battersby,B.J. and Trau,M. (2002) Novel miniaturized systems in high-throughput screening. *Trends Biotechnol.*, 20, 167–173.
6. He,N., Liu,T. and Liu,B. (2016) Technologies and applications in micro-volume liquid handling. *J. Nanosci. Nanotechnol.*, 16, 58–66.
7. Zhu,Y. and Fang,Q. (2013) Analytical detection techniques for droplet microfluidics—a review. *Anal. Chim. Acta*, 787, 24–35.

8. Xiao,H., Bao,Z. and Zhao,H. (2015) High throughput screening and selection methods for directed enzyme evolution. *Ind. Eng. Chem. Res.*, 54, 4011–4020.

9. Lin,J.L., Wagner,J.M. and Alper,H.S. (2017) Enabling tools for high-throughput detection of metabolites: metabolic engineering and directed evolution applications. *Biotechnol. Adv.*, 35, 950–970.

10. Gubler,H. (2016). High-throughput screening data analysis. In: L Zhang (ed). *Nonclinical Statistics for Pharmaceutical and Biotechnology Industries*. Springer International Publishing, Cham, pp. 83–139.

11. Wildey,M.J., Haunso,A., Tudor,M., Webb,M. and Connick,J.H. (2017) High-throughput screening. In: Goodnow R (ed.) *Platform Technologies in Drug Discovery Validation*, First edn, Vol. 50, Academic Press, Cambridge, Massachusetts, 2017, pp. 149–195.

12. Prevel,C., Kurzawa,L., Van,T.N. and Morris,M.C. (2014) Fluorescent biosensors for drug discovery new tools for old targets—screening for inhibitors of cyclin-dependent kinases. *Eur. J. Med. Chem.*, 88, 74–88.

13. Packer,M.S. and Liu,D.R. (2015) Methods for the directed evolution of proteins. *Nat. Rev. Genet.*, 16, 379–394.

14. Longwell,C.K., Labanieh,L. and Cochran,J.R. (2017) High-throughput screening technologies for enzyme engineering. *Curr. Opin. Biotechnol.*, 48, 196–202.

15. Inglese,J., Johnson,R.L., Simeonov,A., Xia,M., Zheng,W., Austin,C.P. and Auld,D.S. (2007) High-throughput screening assays for the identification of chemical probes. *Nat. Chem. Biol.*, 3, 466–479.

16. Janzen,W.P. (2014) Screening technologies for small molecule discovery: the state of the art. *Chem. Biol.*, 21, 1162–1170.

17. Doyle,S.K., Pop,M.S., Evans,H.L. and Koehler,A.N. (2016) Advances in discovering small molecules to probe protein function in a systems context. *Curr. Opin. Chem. Biol.*, 30, 28–36.

18. Biniarz,P., Łukaszewicz,M. and Janek,T. (2017) Screening concepts, characterization and structural analysis of microbial-derived bioactive lipopeptides: a review. *Crit. Rev. Biotechnol.*, 37, 393–410.

19. Sawasaki,T., Ogasawara,T., Morishita,R. and Endo,Y. (2002) A cell-free protein synthesis system for high-throughput proteomics. *Proc. Natl. Acad. Sci. USA*, 99, 14652–14657.

20. Rosenblum,G. and Cooperman,B.S. (2014) Engine out of the chassis: cell-free protein synthesis and its uses. *FEBS Lett.*, 588, 261–268.

21. Zemella,A., Thoring,L., Hoffmeister,C. and Kubick,S. (2015) Cell-free protein synthesis: pros and cons of prokaryotic and eukaryotic systems. *Chembiochem*, 16, 2420–2431.

22. Kanter,G., Yang,J., Voloshin,A., Levy,S., Swartz,J.R. and Levy,R. (2007) Cell-free production of scFv fusion proteins: an efficient approach for personalized lymphoma vaccines. *Blood*, 109, 3393–3399.

23. Fan,J., Villarreal,F., Weyers,B., Ding,Y., Tseng,K.H., Li,J., Li,B., Tan,C. and Pan,T. (2017) Multi-dimensional studies of synthetic genetic promoters enabled by microfluidic impact printing. *Lab Chip*, 17, 2198–2207.

24. Kigawa,T., Yabuki,T., Matsuda,N., Matsuda,T., Nakajima,R., Tanaka,A. and Yokoyama,S. (2004) Preparation of *Escherichia coli* cell extract for highly productive cell-free protein expression. *J. Struct. Funct. Genomics*, 5, 63–68.

25. Katzen,F., Chang,G. and Kudlicki,W. (2005) The past, present and future of cell-free protein synthesis. *Trends Biotechnol.*, 23, 150–156.

26. Carlson,E.D., Gan,R., Hodgman,C.E. and Jewett,M.C. (2012) Cell-free protein synthesis: applications come of age. *Biotechnol. Adv.*, 30, 1185–1194.

27. Dower,W.J. and Mattheakis,L.C. (2002) In vitro selection as a powerful tool for the applied evolution of proteins and peptides. *Curr. Opin. Chem. Biol.*, 6, 390–398.

28. Chandra,H. and Srivastava,S. (2010) Cell-free synthesis-based protein microarrays and their applications. *Proteomics*, 10, 717–730.

29. Kilb,N., Burger,J. and Roth,G. (2014) Protein microarray generation by in situ protein expression from template DNA. *Eng. Life Sci.*, 14, 352–364.

30. Hanes,J. and Plückthun,A. (1997) In vitro selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. USA*, 94, 4937–4942.

31. Roberts,R.W. and Szostak,J.W. (1997) RNA-peptide fusions for the *in vitro* selection of peptides and proteins. *Proc. Natl. Acad. Sci. USA*, 94, 12297–12302.

32. Odegrip,R., Coomber,D., Eldridge,B., Hederer,R., Kuhlman,P.A., Ullman,C., FitzGerald,K. and McGregor,D. (2004) CIS display: *in vitro* selection of peptides from libraries of protein-DNA complexes. *Proc. Natl. Acad. Sci. USA*, 101, 2806–2810.

33. Yamaguchi,J., Naimuddin,M., Biyani,M., Sasaki,T., Machida,M., Kubo,T., Funatsu,T., Husimi,Y. and Nemoto,N. (2009) cDNA display: a novel screening method for functional disulfide-rich peptides by solid-phase synthesis and stabilization of mRNA-protein fusions. *Nucleic Acids Res.*, 37, e108.

34. Mattheakis,L.C., Bhatt,R.R. and Dower,W.J. (1994) An *in vitro* polysome display system for identifying ligands from very large peptide libraries. *Proc. Natl. Acad. Sci. USA*, 91, 9022–9026.

35. Zahnd,C., Amstutz,P. and Pluckthun,A. (2007) Ribosome display: selecting and evolving proteins *in vitro* that specifically bind to a target. *Nat. Methods*, 4, 269–279.

36. Gersuk,G.M., Corey,M.J., Corey,E., Stray,J.E., Kawasaki,G.H. and Vessella,R.L. (1997) High-affinity peptide ligands to prostate-specific antigen identified by polysome selection. *Biochem. Biophys. Res. Commun.*, 232, 578–582.

37. Lamla,T. and Erdmann,V.A. (2003) Searching sequence space for high-affinity binding peptides using ribosome display. *J. Mol. Biol.*, 329, 381–388.

38. Yau,K.Y.F., Groves,M.A.T., Li,S., Sheedy,C., Lee,H., Tanha,J., MacKenzie,C.R., Jermutus,L. and Hall,J.C. (2003) Selection of hapten-specific single-domain antibodies from a non-immunized llama ribosome display library. *J. Immunol. Methods*, 281, 161–175.

39. Gold,L. (2001) mRNA display: diversity matters during *in vitro* selection. *Proc. Natl. Acad. Sci. USA*, 98, 4825–4826.

40. Xu,L., Aha,P., Gu,K., Kuimelis,R.G., Kurz,M., Lam,T., Lim,A.C., Liu,H., Lohse,P.A., Sun,L., Weng,S., Wagner,R.W. and Lipovsek,D. (2002) Directed evolution of high-affinity antibody mimics using mRNA display. *Chem. Biol.*, 9, 933–942.

41. Fukuda,I., Kojoh,K., Tabata,N., Doi,N., Takashima,H., Miyamoto-Sato,E. and Yanagawa,H. (2006) *In vitro* evolution of single-chain antibodies using mRNA display. *Nucleic Acids Res.*, 34, e127.

42. Wilson,D.S., Keefe,A.D. and Szostak,J.W. (2001) The use of mRNA display to select high-affinity protein-binding peptides. *Proc. Natl. Acad. Sci. USA*, 98, 3750–3755.

43. McPherson,M., Yang,Y., Hammond,P.W. and Kreider,B.L. (2002) Drug receptor identification from multiple tissues

using cellular-derived mRNA display libraries. *Chem. Biol.*, 9, 691–698.

44. Takahashi,T.T., Austin,R.J. and Roberts,R.W. (2003) mRNA display: ligand discovery, interaction analysis and beyond. *Trends Biochem. Sci.*, 28, 159–165.

45. Lipovsek,D. and Pluckthun,A. (2004) In-vitro protein evolution by ribosome display and mRNA display. *J. Immunol. Methods*, 290, 51–67.

46. Wang,H. and Liu,R. (2011) Advantages of mRNA display selections over other selection techniques for investigation of protein-protein interactions. *Expert Rev. Proteomics*, 8, 335–346.

47. Patel,S., Mathonet,P., Jaulent,A.M. and Ullman,C.G. (2013) Selection of a high-affinity WW domain against the extracellular region of VEGF receptor isoform-2 from a combinatorial library using CIS display. *Protein Eng. Des. Sel.*, 26, 307–315.

48. Mathonet,P., Ioannou,A., Betley,J. and Ullman,C. (2011) CIS display, a DNA-based *in vitro* selection technology for therapeutic peptides. *Chim. Oggi-Chem. Today*, 29, 10–12.

49. Ueno,S., Yoshida,S., Mondal,A., Nishina,K., Koyama,M., Sakata,I., Miura,K., Hayashi,Y., Nemoto,N., Nishigaki,K. and Sakai,T. (2012) *In vitro* selection of a peptide antagonist of growth hormone secretagogue receptor using cDNA display. *Proc. Natl. Acad. Sci. USA*, 109, 11121–11126.

50. Mochizuki,Y., Biyani,M., Tsuji-Ueno,S., Suzuki,M., Nishigaki,K., Husimi,Y. and Nemoto,N. (2011) One-pot preparation of mRNA/cDNA display by a novel and versatile puromycin-linker DNA. *ACS Comb. Sci.*, 13, 478–485.

51. Ueno,S., Kimura,S., Ichiki,T. and Nemoto,N. (2012) Improvement of a puromycin-linker to extend the selection target varieties in cDNA display method. *J. Biotechnol.*, 162, 299–302.

52. Mochizuki,Y., Kohno,F., Nishigaki,K. and Nemoto,N. (2013) A pull-down method with a biotinylated bait protein prepared by cell-free translation using a puromycin linker. *Anal. Biochem.*, 434, 93–95.

53. ——, Suzuki,T., Fujimoto,K. and Nemoto,N. (2015) A versatile puromycin-linker using cnvK for high-throughput *in vitro* selection by cDNA display. *J. Biotechnol.*, 212, 174–180.

54. Yokoyama,S., Hirota,H., Kigawa,T., Yabuki,T., Shirouzu,M., Terada,T., Ito,Y., Matsuo,Y., Kuroda,Y., Nishimura,Y., Kyogoku,Y., Miki,K., Masui,R. and Kuramitsu,S. (2000) Structural genomics projects in Japan. *Nat. Struct. Biol.*, 7(Suppl), 943–945.

55. Shih,Y.P., Kung,W.M., Chen,J.C., Yeh,C.H., Wang,A.H. and Wang,T.F. (2002) High-throughput screening of soluble recombinant proteins. *Protein Sci.*, 11, 1714–1719.

56. Shin,J., Jardine,P. and Noireaux,V. (2012) Genome replication, synthesis, and assembly of the bacteriophage T7 in a single cell-free reaction. *ACS Synth. Biol.*, 1, 408–413.

57. Hu,S., Xie,Z., Qian,J., Blackshaw,S. and Zhu,H. (2011) Functional protein microarray technology. *Wiley Interdiscip. Rev. Syst. Biol. Med.*, 3, 255–268.

58. He,M. and Taussig,M.J. (2001) Single step generation of protein arrays from DNA by cell-free expression and in situ immobilisation (PISA method). *Nucleic Acids Res.*, 29, 73e.

59. Ueno,S., Biyani,M., Sato,S., Raj Kumal,S., Kuramochi,H., Akagi,T. and Ichiki,T. (2015) Present status and trends in development of high density microarray. *Bunseki Kagaku*, 64, 421–429.

60. He,M., Stoevesandt,O., Palmer,E.A., Khan,F., Ericsson,O. and Taussig,M.J. (2008) Printing protein arrays from DNA arrays. *Nat. Methods*, 5, 175–177.

61. Biyani,M., Moriyasu,J., Tanaka,Y., Sato,S., Ueno,S. and Ichiki,T. (2013) Microintaglio printing of in situ synthesized proteins enables rapid printing of high-density protein microarrays directly from DNA microarrays. *Appl. Phys. Express*, 6, 087001.

62. He,M. and Taussig,M.J. (2003) DiscernArray^TM technology: a cell-free method for the generation of protein arrays from PCR DNA. *J. Immunol. Methods*, 274, 265–270.

63. Angenendt,P., Nyarsik,L., Szaflarski,W., Glökler,J., Nierhaus,K.H., Lehrach,H., Cahill,D.J. and Lueking,A. (2004) Cell-free protein expression and functional assay in nano-well chip format. *Anal. Chem.*, 76, 1844–1849.

64. Ramachandran,N., Hainsworth,E., Bhullar,B., Eisenstein,S., Rosen,B., Lau,A.Y., Walter,J.C. and LaBaer,J. (2004) Self-assembling protein microarrays. *Science*, 305, 86–90.

65. Tao,S.-C. and Zhu,H. (2006) Protein chip fabrication by capture of nascent polypeptides. *Nat. Biotechnol.*, 24, 1253–1254.

66. Ramachandran,N., Raphael,J.V., Hainsworth,E., Demirkan,G., Fuentes,M.G., Rolfs,A., Hu,Y. and LaBaer,J. (2008) Next-generation high-density self-assembling functional protein arrays. *Nat. Methods*, 5, 535–538.

67. Anderson,K.S., Ramachandran,N., Wong,J., Raphael,J.V., Hainsworth,E., Demirkan,G., Cramer,D., Aronzon,D., Hodi,F.S., Harris,L., Logvinenko,T. and LaBaer,J. (2008) Application of protein microarrays for multiplexed detection of antibodies to tumor antigens in breast cancer. *J. Proteome Res.*, 7, 1490–1499.

68. Miersch,S., Bian,X., Wallstrom,G., Sibani,S., Logvinenko,T., Wasserfall,C.H., Schatz,D., Atkinson,M., Qiu,J. and LaBaer,J. (2013) Serological autoantibody profiling of type 1 diabetes by protein arrays. *J. Proteomics*, 94, 486–496.

69. Gibson,D.S., Qiu,J., Mendoza,E.A., Barker,K., Rooney,M.E. and LaBaer,J. (2012) Circulating and synovial antibody profiling of juvenile arthritis patients by nucleic acid programmable protein arrays. *Arthritis Res. Ther.*, 14, R77.

70. Ramachandran,N., Srivastava,S. and Labaer,J. (2008) Applications of protein microarrays for biomarker discovery. *Proteomics Clin. Appl.*, 2, 1444–1459.

71. Diez,P., Gonzalez-Gonzalez,M., Lourido,L., Degano,R.M., Ibarrola,N., Casado-Vela,J., LaBaer,J. and Fuentes,M. (2015) NAPPA as a real new method for protein microarray generation. *Microarrays (Basel)*, 4, 214–227.

72. Angenendt,P., Glökler,J., Konthur,Z., Lehrach,H. and Cahill,D.J. (2003) 3D protein microarrays: performing multiplex immunoassays on a single chip. *Anal. Chem.*, 75, 4368–4372.

73. ——, Kreutzberger,J., Glokler,J. and Hoheisel,J.D. (2006) Generation of high density protein microarrays by cell-free in situ expression of unpurified PCR products. *Mol. Cell Proteomics*, 5, 1658–1666.

74. Konthur,Z. and Wilde,J. (2010). Evaluation of recombinant antibodies on protein microarrays applying the multiple spotting technique. In: R Kontermann and S Dübel (eds). *Antibody Engineering*. Springer, Berlin.

75. Stoevesandt,O., Vetter,M., Kastelic,D., Palmer,E.A., He,M. and Taussig,M.J. (2011) Cell free expression put on the spot: advances in repeatable protein arraying from DNA (DAPA). *N. Biotechnol.*, 28, 282–290.

76. Schmidt,R., Cook,E.A., ——, Taussig,M.J. and Stoevesandt,O. (2013) Optimised 'on demand' protein arraying from DNA by cell free expression with the 'DNA to Protein Array' (DAPA) technology. *J. Proteomics*, 88, 141–148.

77. Biyani,M., Osawa,T., Nemoto,N. and Ichiki,T. (2011) Microintaglio printing of biomolecules and its application to

in situ production of messenger ribonucleic acid display microarray. *Appl. Phys. Express*, 4, 047001.

78. ——, Tanaka,Y., Sato,S., Ueno,S. and Ichiki,T. (2014) Evaluation of poly(dimethylsiloxane) microreactors for pattern size miniaturization of microintaglio-printing-based protein microarray. *Jpn. J. Appl. Phys.*, 53, 06JL04.

79. Kobayashi,R., Biyani,M., Ueno,S., Kumal,S.R., Kuramochi,H. and Ichiki,T. (2015) Temperature-controlled microintaglio printing for high-resolution micropatterning of RNA molecules. *Biosens. Bioelectron.*, 67, 115–120.

80. Kim,S.H., Yoshizawa,S., Takeuchi,S., Fujii,T. and Fourmy,D. (2013) Ultra-high density protein spots achieved by on chip digitalized protein synthesis. *Analyst*, 138, 4663–4669.

81. Buxboim,A., Bar-Dagan,M., Frydman,V., Zbaida,D., Morpurgo,M. and Bar-Ziv,R. (2007) A single-step photolithographic interface for cell-free gene expression and active biochips. *Small*, 3, 500–510.

82. Bar,M. and Bar-Ziv,R.H. (2009) Spatially resolved DNA brushes on a chip: gene activation by enzymatic cascade. *Nano Lett.*, 9, 4462–4466.

83. Heyman,Y., Buxboim,A., Wolf,S.G., Daube,S.S. and Bar-Ziv,R.H. (2012) Cell-free protein synthesis and assembly on a biochip. *Nat. Nanotechnol.*, 7, 374–378.

84. Tawfik,D.S. and Griffiths,A.D. (1998) Man-made cell-like compartments for molecular evolution. *Nat. Biotechnol.*, 16, 652–656.

85. Bernath,K., Hai,M., Mastrobattista,E., Griffiths,A.D., Magdassi,S. and Tawfik,D.S. (2004) in vitro compartmentalization by double emulsions: sorting and gene enrichment by fluorescence activated cell sorting. *Anal. Biochem.*, 325, 151–157.

86. Fujii,S., Matsuura,T., Sunami,T., Nishikawa,T., Kazuta,Y. and Yomo,T. (2014) Liposome display for *in vitro* selection and evolution of membrane proteins. *Nat. Protoc.*, 9, 1578–1591.

87. Doi,N. and Yanagawa,H. (1999) STABLE: protein-DNA fusion system for screening of combinatorial protein libraries *in vitro*. *FEBS Lett.*, 457, 227–230.

88. Yonezawa,M. (2003) DNA display for *in vitro* selection of diverse peptide libraries. *Nucleic Acids Res.*, 31, 118e–1118.

89. Ghadessy,F.J., Ong,J.L. and Holliger,P. (2001) Directed evolution of polymerase function by compartmentalized self-replication. *Proc. Natl. Acad. Sci. USA*, 98, 4552–4557.

90. Sepp,A., Tawfik,D.S. and Griffiths,A.D. (2002) Microbead display by *in vitro* compartmentalisation: selection for binding using flow cytometry. *FEBS Lett.*, 532, 455–458.

91. Levy,M., Griswold,K.E. and Ellington,A.D. (2005) Direct selection of trans-acting ligase ribozymes by *in vitro* compartmentalization. *RNA*, 11, 1555–1562.

92. Kelly,B.T. and Griffiths,A.D. (2007) Selective gene amplification. *Protein Eng. Des. Sel.*, 20, 577–581.

93. Stein,V., Sielaff,I., Johnsson,K. and Hollfelder,F. (2007) A covalent chemical genotype-phenotype linkage for *in vitro* protein evolution. *Chembiochem*, 8, 2191–2194.

94. Mastrobattista,E., Taly,V., Chanudet,E., Treacy,P., Kelly,B.T. and Griffiths,A.D. (2005) High-throughput screening of enzyme libraries: *in vitro* evolution of a β-galactosidase by fluorescence-activated sorting of double emulsions. *Chem. Biol.*, 12, 1291–1300.

95. Fen,C.X., Coomber,D.W., Lane,D.P. and Ghadessy,F.J. (2007) Directed evolution of p53 variants with altered DNA-binding specificities by *in vitro* compartmentalization. *J. Mol. Biol.*, 371, 1238–1248.

96. Hardiman,E., Gibbs,M., Reeves,R. and Bergquist,P. (2010) Directed evolution of a thermophilic beta-glucosidase for cellulosic bioethanol production. *Appl. Biochem. Biotechnol.*, 161, 301–312.

97. Korfer,G., Pitzler,C., Vojcic,L., Martinez,R. and Schwaneberg,U. (2016) *In vitro* flow cytometry-based screening platform for cellulase engineering. *Sci. Rep.*, 6, 26128.

98. Ostafe,R., Prodanovic,R., Commandeur,U. and Fischer,R. (2013) Flow cytometry-based ultra-high-throughput screening assay for cellulase activity. *Anal. Biochem.*, 435, 93–98.

99. ——, ——, Nazor,J. and Fischer,R. (2014) Ultra-high-throughput screening method for the directed evolution of glucose oxidase. *Chem. Biol.*, 21, 414–421.

100. Stapleton,J.A. and Swartz,J.R. (2010) Development of an *in vitro* compartmentalization screen for high-throughput directed evolution of [FeFe] hydrogenases. *PLoS One*, 5, e15275.

101. Tu,R., Martinez,R., Prodanovic,R., Klein,M. and Schwaneberg,U. (2011) A flow cytometry-based screening system for directed evolution of proteases. *J. Biomol. Screen.*, 16, 285–294.

102. Gupta,R.D., Goldsmith,M., Ashani,Y., Simo,Y., Mullokandov,G., Bar,H., Ben-David,M., Leader,H., Margalit,R., Silman,I., Sussman,J.L. and Tawfik,D.S. (2011) Directed evolution of hydrolases for prevention of G-type nerve agent intoxication. *Nat. Chem. Biol.*, 7, 120–125.

103. Hwang,B.-Y. (2012) Directed evolution of cutinase using *in vitro* compartmentalization. *Biotechnol. Bioprocess Eng.*, 17, 500–505.

104. Kintses,B., Hein,C., Mohamed,M.F., Fischlechner,M., Courtois,F., Laine,C. and Hollfelder,F. (2012) Picoliter cell lysate assays in microfluidic droplet compartments for directed enzyme evolution. *Chem. Biol.*, 19, 1001–1009.

105. Houlihan,G., Gatti-Lafranconi,P., Kaltenbach,M., Lowe,D. and Hollfelder,F. (2014) An experimental framework for improved selection of binding proteins using SNAP display. *J. Immunol. Methods*, 405, 47–56.

106. ——, ——, Lowe,D. and Hollfelder,F. (2015) Directed evolution of anti-HER2 DARPins by SNAP display reveals stability/function trade-offs in the selection process. *Protein Eng. Des. Sel.*, 28, 269–279.

107. Petrie,K.L. and Joyce,G.F. (2014) Limits of neutral drift: lessons from the *in vitro* evolution of two ribozymes. *J. Mol. Evol.*, 79, 75–90.

108. Takeuchi,R., Choi,M. and Stoddard,B.L. (2014) Redesign of extensive protein-DNA interfaces of meganucleases using iterative cycles of *in vitro* compartmentalization. *Proc. Natl. Acad. Sci. USA*, 111, 4061–4066.

109. Gianella,P., Snapp,E.L. and Levy,M. (2016) An *in vitro* compartmentalization-based method for the selection of bond-forming enzymes from large libraries. *Biotechnol. Bioeng.*, 113, 1647–1657.

110. Sepp,A. and Choo,Y. (2005) Cell-free selection of zinc finger DNA-binding proteins using *in vitro* compartmentalization. *J. Mol. Biol.*, 354, 212–219.

111. Bernath,K., Magdassi,S. and Tawfik,D.S. (2005) Directed evolution of protein inhibitors of DNA-nucleases by *in vitro* compartmentalization (IVC) and nano-droplet delivery. *J. Mol. Biol.*, 345, 1015–1026.

112. Agresti,J.J., Kelly,B.T., Jäschke,A. and Griffiths,A.D. (2005) Selection of ribozymes that catalyse multiple-turnover Diels–Alder cycloadditions by using *in vitro* compartmentalization. *Proc. Natl. Acad. Sci. USA*, 102, 16170–16175.

113. Zaher,H.S. and Unrau,P.J. (2007) Selection of an improved RNA polymerase ribozyme with superior extension and fidelity. *RNA*, 13, 1017–1026.

114. Zheng,Y. and Roberts,R.J. (2007) Selection of restriction endonucleases using artificial cells. *Nucleic Acids Res*., 35, e83.

115. Chen,Y., Mandic,J. and Varani,G. (2008) Cell-free selection of RNA-binding proteins using *in vitro* compartmentalization. *Nucleic Acids Res*., 36, e128.

116. Levy,M. and Ellington,A.D. (2008) Directed evolution of streptavidin variants using *in vitro* compartmentalization. *Chem. Biol*., 15, 979–989.

117. Tay,Y., Ho,C., Drőge,P. and Ghadessy,F.J. (2010) Selection of bacteriophage λ integrases with altered recombination specificity by *in vitro* compartmentalization. *Nucleic Acids Res*., 38, e25.

118. Prodanovic,R., Ostafe,R., Blanusa,M. and Schwaneberg,U. (2012) Vanadium bromoperoxidase-coupled fluorescent assay for flow cytometry sorting of glucose oxidase gene libraries in double emulsions. *Anal. Bioanal. Chem*., 404, 1439–1447.

119. Paul,S., Stang,A., Lennartz,K., Tenbusch,M. and Uberla,K. (2013) Selection of a T7 promoter mutant with enhanced *in vitro* activity by a novel multi-copy bead display approach for *in vitro* evolution. *Nucleic Acids Res*., 41, e29.

120. Ma,F., Xie,Y., Huang,C., Feng,Y. and Yang,G. (2014) An improved single cell ultrahigh throughput screening method based on *in vitro* compartmentalization. *PLoS One*, 9, e89785.

121. Shimizu,Y., Inoue,a., Tomari,Y., Suzuki,T., Yokogawa,T., Nishikawa,K. and Ueda,T. (2001) Cell-free translation reconstituted with purified components. *Nat. Biotechnol*., 19, 751–755.

122. Ohta,N., Kato,Y., Watanabe,H., Mori,H. and Matsuura,T. (2016) *In vitro* membrane protein synthesis inside Sec translocon-reconstituted cell-sized liposomes. *Sci. Rep*., 6, 36466.

123. Uyeda,A., Watanabe,T., Kato,Y., Watanabe,H., Yomo,T., Hohsaka,T. and Matsuura,T. (2015) Liposome-based *in vitro* evolution of aminoacyl-tRNA synthetase for enhanced pyrrolysine derivative incorporation. *Chembiochem*, 16, 1797–1802.

124. Fujii,S., Matsuura,T. and Yomo,T. (2015) *In vitro* directed evolution of alpha-hemolysin by liposome display. *Biophysics (Nagoya-Shi)*, 11, 67–72.

125. Villarreal,F., Contreras-Llano,L.E., Chavez,M., Ding,Y., Fan,J., Pan,T. and Tan,C. (2018) Synthetic microbial consortia enable assembly of pure translation machinery. *Nat. Chem. Biol*., 14, 29–35.