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Recent developments in nucleic acid identification using solid-phase enzymatic assays

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Abstract

This review covers recent achievements in the development of new approaches for enzymatically assisted detection of nucleic acids on microarrays. We discuss molecular techniques including polymerase chain reaction, reverse transcription, allele specific primer extension and a range of isothermal amplification techniques for the amplification and discrimination of nucleic acids. This also includes their implementation in microfluidic-based systems. These techniques all show great promise for use in the life sciences allowing for high throughput, cost effective and highly sensitive and specific nucleic acid analysis. Importantly they can be potentially integrated into personalized and point-of-care medicine.

Includes 103 references.

Graphical abstract text

This review highlights recent developments in molecular techniques including polymerase chain reaction, reverse transcription, allele specific primer extension and isothermal amplification techniques for the amplification and discrimination of nucleic acids. Their use in microfluidic platforms is also discussed.

Keywords

Solid-phase PCR, Microarray, Nucleic Acid Amplification, Isothermal Amplification, Microfluidics
Introduction

Microarray technologies, introduced more than two decades ago, have been the starting point for a variety of high throughput molecular bioanalytical methods enabling fundamental breakthroughs in both the life sciences and clinical diagnostics. Microarrays are typically constructed of glass, polymer or silicon wafers, upon which molecular probes (DNA, protein, antibodies, carbohydrates etc) are spatially deposited, or directly synthesized. This allows for a predetermined order that permits multiple simultaneous independent analyses, thus achieving multi-parametric screening. Since their introduction microarrays have become an increasingly important field of research, witnessed by the large number of papers published on the development of microarrays for analysis of DNA, proteins and cells.

More recently attention has been drawn to the use of “active arrays” [1, 2] which offer the possibility of performing different enzymatic reactions with immobilized biomolecules on a solid support, thus providing additional sensitivity, specificity and scalability of analysis. The most successful implementation of active arrays has been in the development of high throughout DNA sequencing (HTS), also known as next generation sequencing (NGS). NGS has resulted in the rapid (under 2 weeks) sequencing of the human genome (for review see [3–5]). In general all current HTS technologies are essentially highly evolved versions of the classic DNA hybridization microarrays first proposed by Southern in 1992 [6].

In the following we review the most recent achievements (within the last 6-7 years) in the development, and application, of microarrays for “active” quantitative and qualitative identification of nucleic acids by means of both PCR-like techniques and isothermal amplification. The implementation of microfluidic-base microarrays and arrays made by using hydrophobic-hydrophilic patterns will also be discussed. Finally solid-phase nucleic acid amplification as a highly efficient signal enhancement technique for protein and cell microarrays and some new approaches for integrated active array detection systems will be highlighted.

Approaches for solid-phase DNA amplification

Microarray DNA (primer) immobilization

The immobilization of primers onto a solid-phase support for subsequent amplification is a challenging task. To do this the immobilized primers should have a strong link between the primer and microarray support, high surface coverage, and have an appropriate lateral spacing in order to reduce steric hindrance while interacting with complementary DNA/RNA strands and enzyme molecules. Thus, selection of the microarray solid support material and primer immobilization chemistries is highly important. To this end a popular approach is to first
modify the surface with amino silane reagents which results in a uniform layer of covalently attached primary amines. These can then be easily functionalized to the appropriate activated group for reaction with amino-, phosphate- or acrylamide-modified DNA [7–9]. Highly selective copper-catalyzed alkyne–azide cycloaddition (CuCAAC) has also been demonstrated for microarray DNA immobilization [10] while non-covalent attachment can be employed using biotin-avidin interactions [11] or magnetically functionalized primers [12, 13]. These approaches allow for the high density immobilization of DNA molecules, however, do not provide uniform and controlled spacing between them. Recently there has been a breakthrough in the lateral control of immobilized DNA through the use of cone-shaped nano-metric dendron molecules of uniform size (Fig. 1A) [14–16]. It has been shown that DNA immobilization through dendrons provides appropriate lateral spacing and surface orientation of the DNA primers which results in an eight fold increase in the fluorescent signal after enzymatic DNA polymerization when compared with those based on high density amine and aldehyde slides (Fig. 1B) [15].

![Fig. 1](A) Illustration of dendron molecules functionalized with an aldehyde group and its incorporation onto the surface of a glass slide; reproduced from [14] and (B) Efficiency of the hybridization and on-chip DNA polymerization; reproduced from [15].

Work reported by the Wolff group has demonstrated an approach to the covalent immobilization of specific primers tagged at the 5’ termini with a homopolymer sequence polyT10-polyC10 (TC tag) on unmodified glass slides using simple UV cross-linking [17–19]. This resulted in a more stable covalent attachment to the glass surface which sometimes can be an issue in solid-phase amplification [18, 20].

A method that has attracted a lot of attention is one developed by Mirzabekov et al [21, 22] who immobilized DNA within a three-dimensional acrylamide gel. The structure of the individual gel elements provided an increased loading capacity and an enzyme friendly liquid environment. This improved the
performance of hybridization and various enzymatic reactions such as quantitative solid-phase PCR [23–25], primer extension [20, 26] and pyrosequencing [20].

**Polymerase chain reaction (PCR) on microarrays**

Historically PCR has been one of the first examples of the use of enzymatic reactions on DNA microarrays [27]. Accordingly, there has been a plethora of applications on its implementation on microarrays for qualitative and quantitative nucleic acids analysis [23, 28–31], species discrimination [23, 32, 33], point mutations and single nucleotide polymorphs (SNPs) [34–37] and gene expression analysis [2, 38]. The design of the approach is very straightforward whereby a PCR primer is covalently tethered to a solid-phase support, while the remaining components of the PCR mixture which include an opposite primer, DNA polymerase, buffer constituents and target DNA are present in the solution covering the microarray. At the beginning of the reaction asymmetric amplification of the target DNA usually initiates in the liquid phase. This occurs due to the high non-stoichiometric ratio of the liquid-phase primers to those immobilized on the solid-phase along with hindered diffusion of the DNA molecules to the surface immobilized primers [24]. Subsequently, newly synthesized DNA strands anneal to the solid-phase immobilized primers which in turn are enzymatically extended by a DNA polymerase forming a double-stranded PCR (ds-PCR) product covalently tethered to the surface (Fig. 2 (A-D)). The addition to the solution of a small amount, usually less than 10%, of the same primer as immobilized on the solid-support allows for increased efficacy of the amplification [3, 13, 17, 18].

![Diagram](image)

**Fig. 2** Schematic representation of a PCR amplification performed on solid surface with immobilized primers. Initially, the reaction compartment comprises of solid-phase primers as well as forward (fwd) and reverse (rev) primers in an asymmetric ratio (A); at the start of the reaction the PCR proceeds preferably in the liquid phase, until the forward primer is depleted (B); then the solid-phase PCR dominates where the immobilized primer is
extended by DNA polymerase (C) and (D). Adapted from [39] with permission from The Royal Society of Chemistry.

**Solid-phase amplification on two-dimensional microarrays**

After immobilization of the PCR primers to a solid support PCR amplification can then be performed. Spatial separation of PCR primers on various microarray elements significantly minimizes undesirable primer interactions hence reducing the formation of primer dimers, increasing specificity and sensitivity of amplification, and allowing for highly multiplexed analyses [40, 41]. For example, Hoffmann and co-workers successfully demonstrated highly parallel solid-phase PCR within 19 pL wells of a picowell array containing $1.10 \times 10^5$ individual reaction spots (vessels) (Roche Diagnostics, Germany) with immobilized primers [40]. Specific primers were first immobilized on a polydimethylsiloxane (PDMS) substrate then the substrate was covered with the picowell array containing the $1.10 \times 10^5$ individual reaction spots (vessels). This gave spatial separation of the immobilized primers on the microarray as well as leak-proof sealing between the array and the PDMS. PCR was then shown to occur within 97.2% of the spots (Fig. 3(A-C)). The lengths of the PCR products amplified were in the range of 100 to 1513 base pairs (bp) showing no significant dependency between the solid-phase amplification efficiency and the length of the PCR products.

![Fig. 3](image)

**Fig. 3** Direct detection of solid-phase amplification by molecular hybridization. (A) The specificity of the reaction was detected by hybridizing a Cy3-labeled probe against a solid-phase bound 1513 bp PCR product, (B) Indirect detection by reacting streptavidin-Cy5 with biotin molecules which have been incorporated into the 346 bp template during solid-phase primer extension reactions and (C) Digital solid-phase PCR: the reaction mix contained < 1 template molecule per well each of the 346 bp and 1513 bp PCR products. The reaction was detected by hybridization. The green colour represents the 1513 bp PCR product, the red colour the 346 bp PCR product, and the yellow colour mixed signals from the 1513 bp and 346 bp PCR products. Scale bars are 200 $\mu$m (A and B) and 100 $\mu$m (C). Adapted from [40] with permission from The Royal Society of Chemistry.
Solid-phase amplification systems with nested primers have also been described [16, 17, 19, 33]. Usually such systems consist of two freely floating primers (forward and reverse) and one immobilized nested primer used to increase efficacy and provide additional specificity of amplification. To this end Kim et al. [16] have designed microarrays for human papilloma virus (HPV) genotyping in which two solution-based primers were responsible for the “bulk” amplification of the L1 region of the HPV genome, while nested immobilized primers were used for HPV genotyping. The authors found that the best single nucleotide discrimination was achieved when the discriminating nucleotide was located three or four bases from its 3’ termini (Fig. 4). This distinguishes the approach from standard allele specific PCR where the discrimination nucleotide is usually placed at the 3’ end of the allele specific primer [42, 43].

![Image](image.jpg)

**Fig. 4** Efficacy of allele specific solid-phase amplification depending on the location of the discriminating nucleotide within immobilized primers. The discriminating nucleotide was placed at the 3’ termini of the immobilized primer – (A), at the second position from the 3’ termini – (B), at the third position from the 3’ termini - (C), and at the fourth position from the 3’ termini - (D). The upper line in each image corresponds to fully complementarity of the primer and target DNA. Reproduced from [16].

Pierik and co-workers [30] demonstrated the approach described by Kim et al [16] for real-time quantitative solid-phase hybridization performed along with PCR amplification. In their approach the authors immobilized specific nested capture probes through the 3’ termini rather than through the 5’ termini, as is normally used for solid-phase PCR. During the PCR amplification, conducted under “normal conditions” namely with two liquid-phase primers one of which was fluorescently labelled, the authors registered the hybridization of the amplification products to the immobilized capture probes at the annealing stage. Real-time
monitoring of the hybridization kinetics allowed for a classic S-shaped qPCR amplification curve to be obtained. As a model system methicillin-sensitive Staphylococcus epidermidis (MSSE) femA gene was used. The lowest detectable concentration of 25 gEq (genome-equivalents) per assay was successfully achieved; however, the amplification efficacy was only 58% [10]. An interesting aspect of this research was the use of “confocal fluorescent scanning” for real-time hybridization monitoring that permitted signal acquisition with a high signal-to-noise ratio even in the presence of excess unreacted fluorescently labelled primers.

An analogous amplification scheme with 5’-immobilized nested primers was successfully performed for reverse transcription PCR amplification (RT-PCR) which was targeted towards the identification and genotyping of avian influenza viral RNA [17, 19]. In this approach the authors converted target RNA into complementary DNA strands (cDNA) using liquid-phase primers in the solution covering the array. Subsequently the cDNA obtained was PCR amplified and distinguished using different specific nested primers immobilized on the array [19].

Von Nickisch-Rosenegk et al [2] have also shown a reverse transcription reaction on a microarray immobilized with a primer followed by subsequent solid-phase PCR for gene expression analysis. In their work there appeared to be no significant difference in the gene expression performance whether a two- (ImPromII reverse transcriptase for reverse transcription, and Taq DNA polymerase for cDNA amplification) or a one-enzyme (rTth DNA polymerase for both reverse transcription and cDNA amplification) amplification system was employed [2].

An original approach describing a solid-phase amplification with only one surface immobilized primer, and in the absence of a liquid-phase primer, has been described by Kinoshita et al [44]. In this work they tethered a single primer to a solid surface covered with the biocompatible phospholipid polymer, poly[2-methacryloyloxyethyl phosphorylcholine (MPC)-co-n-butyl methacrylate (BMA)-co-p-nitrophenyloxycarbonyl polyethylene glycol methacrylate (MEONP)] (PMBN). As a model target a 50 nucleotide long synthetic DNA was used. The workflow consisted of 30 repetitive cycles of hybridization of the DNA target to the immobilized primers which were then elongated with Taq DNA polymerase followed by thermal denaturation [44]. The detection of this primer extension was monitored by the incorporation of Cy-3 labelled dUTP’s. This involved a process where during the annealing stage of each cycle the DNA target molecules annealed to the new unextended primers resulting in new elongation and incorporation of the Cy-3-dUTP. The lowest detection limit of 1 pM was achieved. Most likely the high sensitivity was due to the biocompatibility of the polymer support which provided an “ideal” environment for maintaining the activity of the DNA polymerase and the short length
of the synthetic target ssDNA molecule. Subsequently, the proposed approach was implemented for real-life SNP genotyping (polymorphism A118G in the human µ opioid receptor gene) [45]. However, the sensitivity of the developed method was not enough to discriminate SNPs from human genomic DNA samples and a preliminary PCR amplification step was required.

*Solid-phase amplification within three-dimensional microarray elements*

Seminal work in the development of solid-phase PCR amplification within acrylamide gel pads for downstream enzymatic applications has been demonstrated by Haung et al [20]. The authors report on the successful hybridization- and enzymatic-based SNP genotyping of the gel immobilized solid-phase PCR products of the 14417 SNP locus of the Ox-LDL receptor 1 (OLR-1) gene. After solid-phase amplification the ds-PCR products were denatured and the complementary DNA strand was removed from the gel pad via electrophoresis under alkaline conditions. This resulted in single-stranded DNA (ss-DNA) immobilized within the gel element. Subsequent application of dual-colour labelled hybridization probes (Fig. 5-1) was used for the hybridization-based SNP genotyping approach. In a second case the ss-DNA amplification product was successfully used for BAMPER (Bioluminometric Assay coupled with Modified Primer Extension Reactions) SNP genotyping [46] (Fig. 5-2). The BAMPER approach is a modified version of an allele specific primer extension reaction with bioluminescent detection of pyrophosphate which is released during the extension reaction [46]. In a final approach DNA pyrosequencing has been demonstrated within microarray gel-pads (Fig. 5-3). These examples clearly show the universality of gel-pad “active” microarrays for a broad range of enzymatic reactions with DNA.
More recently quantitative real-time amplification within hydrogel pads has been demonstrated [23–25]. Identification and kinetic monitoring of the amplification reaction was carried out using nonspecific SYBR Green I dye that binds to the ds-PCR products tethered within the hydrogel pads. Multiplex quantitative analysis of human immunodeficiency virus type 1 (HIV-1) and hepatitis B and C viruses (HBV and HCV) [23] and herpes simplex viruses types 1 and 2, cytomegalovirus DNA, *Chlamydia trachomatis*, *Mycoplasma hominis*, and *Ureaplasma urealyticum* [25] was successfully performed. However the diffusion of PCR products towards the microarray surface and especially within hydrogel elements, is a key factor responsible for the difference in amplification efficacy between solid-phase amplification and traditional liquid phase PCR reactions [24]. Drobyshev et al [24] showed that prolonged incubation times at each stage of solid-phase amplification (and especially at the denaturation step) along with a reduction in reaction volume covering the microarray gel element led to more efficient solid-phase amplification. Further to overcome the limited diffusion issues, and low loading capacity of two dimensional array elements [47], the use of micrometre-sized beads (microbeads) and nanoparticles possessing high surface-to-volume ratios and more efficient surface diffusion has been reported [11, 48–51].

Qi et al [48] and Deng et al [49] described a DNA genotyping system based on an emulsion solid-phase amplification followed by primer extension on microarrays. After the emulsion solid-phase amplification [52, 53] the microbeads with immobilized PCR products were embedded in a polyacrylamide gel and soaked in the reaction mixture for the allele specific primer extension. The use of the proposed approach allowed for quantification of expression levels of multiple colorectal cancer (CRC)-related genes [48]. An approach for immobilization of allele specific primers on magnetic nanoparticles for multi-colour multiple gene SNP genotyping by a primer extension reaction has also been demonstrated [26, 49].

**Microarray solid-phase PCR as a preliminary amplification step for further enzymatic analysis**

Often amplification on solid-phase microarrays is used as a preliminary step for other discriminative enzymatic assays [35–37, 40]. For example, reliable SNP genotyping by solid-phase PCR followed by primer extension has been demonstrated by Brugere et al [36]. After chemical denaturation of the surface tethered ds-PCR products the authors used α-thio-deoxynucleotide triphosphates for the primer extension step followed by an Exonuclease III (ExoIII) treatment. Non-extended primers were shown to hydrolyze whereas the extended primers resisted the ExoIII treatment, indicating which nucleotide had been incorporated [17]. Zhu et al [37] described a label free single base extension (SBE) reaction for SNP genotyping. In their work the authors used
matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to distinguish the extended primers from the non-extended primers.

**Isothermal amplification on microarrays**

Opposite to PCR amplification, which requires precision in thermo-cycling, the isothermal amplification is performed at constant temperature driven by the specific activity of special enzymes. Additionally, isothermal amplification provides fewer requirements to the experimental setup, is more energy efficient and can be adapted to micro-total-analysis system (μTAS) technology for in-field nucleic acid testing. When combined with microarray technology isothermal amplification can potentially open up new horizons as a low-cost, highly sensitive diagnostic tool for home use and/or personalized medicine. In particular, rolling circle amplification (RCA)[54], loop mediated isothermal amplification (LAMP) [55], helicase dependent amplification (HDA) [56] and strand displacement amplification [57–59]) are the most widely used techniques for isothermal DNA/RNA amplification. RCA is one of the simplest isothermal amplification processes in which one single-strand primer is extended by DNA polymerase with strand displacement activity using a circular DNA as a template. The reaction results in long, usually several thousand base single-stranded RCA (ss-RCA) products comprising of repetitive DNA sequences complementary to the circular template used. The simplicity of this reaction makes it very useful for both the detection of nucleic acids, including genotyping, and as an auxiliary reporter method.

Immobilization of the primer for RCA onto solid supports or microbeads [60][61] easily transforms the reaction into the solid-phase regime (Fig. 6). By utilizing primers immobilized on microbeads attomole detection of target circular DNA has been reported [62, 63]. The use of LAMP [55] and HDA [56] methods on solid supports has also been successfully shown.

In general imaging of isothermal amplification products can be achieved by using all the various DNA visualization methods employed in microarray technologies such as hybridization with fluorescently labelled probes [63, 64], chemiluminescent detection [65, 66] and unspecific fluorescent dyes [11, 78].


An interesting approach for detection of ds-DNA using solid-phase RCA has been proposed by Schopf et al [64]. Microbeads functionalized with oligonucleotide capture probes were mixed with ds-DNA targets and linear RCA padlock probes with subsequent denaturing. During annealing to room temperature sequential hybridization of the capture probes, ss-DNA targets as well as the padlock probes occurred, resulting in the ligation of the padlock probes and triggering of the RCA. Subsequently, the microbead immobilized RCA products were hybridized with fluorescent probes and imaged. The proposed approach was successfully implemented for the detection of *M. Tuberculosis* DNA with the sensitivity of 10^4 colonies forming unit per mL.

Isothermal detection of methicillin resistant *S. aureus* (MRSA) and *N. gonorrhoeae* dsDNA has been carried out using HDA [56]. HDA is very similar to PCR amplification with the exception that HDA is performed at a constant temperature. This is due to the fact that the helicase enzyme unwinds ds-DNA thus eliminating the need for thermal denaturation normally employed during PCR thermo-cycling. Shin et al [69] have demonstrated one of the most sophisticated and sensitive approaches for isothermal PCR-like amplification of ds-DNA using a solid-phase immobilized primer. This was carried out using recombinase polymerase amplification (RPA) [70, 71] which employs a uvsX recombinase enzyme of specific activity isolated from T4 phage. In this case the enzyme is intended to “substitute” the thermal denaturation step normally performed during PCR amplification. The enzyme along with single stranded binding proteins facilitates the annealing of

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**Fig. 6** Schematic representation of solid-phase RCA for SNP discrimination. (A) Template, or padlock probe, is perfectly complementary at the 3’ and 5’ termini to the primer sequence. Ligase circularizes the template, and the polymerase enzyme extends the primer in a rolling circle replication reaction. (B) The template strand is not perfectly complementary, at the 3’ and 5’ termini, to the DNA primer sequence. Ligation fails, and polymerase extends the primer to complement the linear template. Reproduced from [103].
the primers to their complementary sequences within the target ds-DNA by means of homologous recombination. Further, primer extension was performed with DNA polymerase showing strand displacement activity. Cyclic repetition of the process led to an exponential amplification of the targeted DNA. Another distinctive feature of the developed method was the use of a label-free detection system based on the wavelength shift of a silicon micro-ring optical resonator [69]. This allowed the amplification signal to be monitored in real-time providing a lower detection limit of 500 fg×µL\(^{-1}\).

Isothermal RCA has also been very popular as a signal amplification method for cell and protein arrays [66, 68, 72, 73]. The strategy for RCA detection of cells or proteins is very similar. For this a RCA primer is first conjugated with an antibody which then binds to a secondary antibody that is captured (sandwich assay) or immobilized on a target protein molecule or cell on a microarray. As an alternative to antibodies the use of DNA/RNA aptamers has also been reported (Fig. 7) [66, 73]. In this method RCA starts in the presence of circular DNA and other reaction components. The detection of the RCA products can be performed using surface associated methods such as microbead assisted diffractometry detection [78] and surface enhanced Raman spectroscopy [72]. Thus single protein recognition events are amplified thousands of times reaching a system sensitivity of 10 zeptomolar (1000 protein molecules) [72].

![Fig. 7](image.png)

**Fig. 7** Schematic representation of an RCA-based microbead detection assay in combination with aptamers. A biotinylated anti-PDGF-B specific aptamer is immobilized on streptavidin-coated periodic patterns. PDGF-B is introduced and then captured by the aptamer. An aptamer-primer complex with an additional primer sequence binds to the protein. A padlock probe hybridized to the primer tail of the aptamer is ligated, and RCA is initiated. Streptavidin-conjugated beads bind to elongated concatamers via hybridized biotinylated probes. Reproduced from [73].
Finally, femtolar concentrations of ss-DNA have been detected using isothermal in-situ RNA transcription-based amplification and SPR detection [74]. The sensing element consisted of two microarray spots with immobilised DNA. The first spot (generator) was immobilized with a capture probe containing a T7 RNA polymerase promoter sequence (Fig. 8). In this particular spot, after hybridization with target DNA, the in-situ RNA transcription occurred. Immediately after this the newly synthesized ss-RNA molecules diffused to the second sensing spot (detector) and hybridized to a second capture probe where they were then detected with nanoparticle enhanced SPR (Fig. 8). The sensitivity of this approach reached 1fM [74].

![Fig. 8 Schematic representation of the solid-phase in situ RNA transcription-based amplification and nanoparticle-enhanced SPR Imaging. (a) On the generator elements a surface promoter DNA is covalently attached to the gold surface and then is base-paired with template DNA from solution. (b) The surface is exposed to a solution of T7 RNA polymerase and rNTPs to produce multiple copies of ss-RNA. (c) The ss-RNA reaches the detector elements through diffusion in the solution, hybridizes with the detector DNA capture probes and then is detected with nanoparticle-enhanced SPR (d). Adapted from [74].](image)

**Microfluidic arrays**

The rapidly growing field of soft-lithography driven microfluidic technology has resulted in the development a plethora of micro-devices for extraction [75] and amplification and analysis [31, 76–81] of nucleic acids. The main idea of microarray technology, namely spatial separation of microarray elements and their simultaneous subjection to one sample of interest can be easily realized using highly parallel architectures of micrometre-sized channels available/affordable due to microfluidics. Parallel architectures of multiple microchannels allow not only covalent immobilization of oligonucleotides in separate channels but, for example, simple physical confinement of microbeads with immobilized primers. Such an approach for SNP genotyping has been shown
by Zhang et al [77]. In their work the authors showed the development of a weir-like microchannel structure with trapped primer immobilized microbeads (Fig. 9). The designed microfluidic confined bead array was successfully demonstrated for apyrase assisted allele-specific primer extension [20, 82]. This approach showed reliable discrimination of synthetic target DNA of 0.5 pM concentration that corresponded to an absolute detection sensitivity of approximately one attomole of target molecules in a 15 µL sample. Similar solution based SNP analysis could detect only 0.1 nM synthetic DNA target molecules [77]. The improved sensitivity was due to the enhanced mass transport, reduced diffusion distance within the microfluidic channel and the high surface-to-volume ratio of the microbeads.

Another example of PCR amplification within acrylamide gel pads (fabricated using soft lithography) with “pseudo immobilized” PCR reagents was demonstrated by Manage et al [83]. The authors performed amplification and melting curve analysis in free standing gel posts (pillar like gel elements with a volume of 0.67 µL) containing all components of the PCR reaction including specific primers for Herpes Simplex Virus (HSV) genotype discrimination and an intercalating dye for fluorescent detection of amplification products. Discrimination between HSV-1 and HSV-2 genotypes was carried out by melting curve analysis only, since kinetic amplification data did not allowed for the discrimination. A year later the same authors [56] reported on a further improvement of the approach and development of the ready-to-use cassette containing short glass capillaries filled with desiccated (for shelf storage) acrylamide gels with all the reagents required for the PCR reaction except the DNA template. In order to perform the nucleic acids testing reaction the cassette has to be filled with raw sample containing the target templates and thermo-cycled.
Fig. 9 (A) Schematic representation of chip fabrication. The arrayed chambers (100 x 100 x 30 µm) were aligned with corresponding bead-loading channels (100 x 30 µm) that contained two disconnected channel segments. The functionalized beads were introduced into the respective bead-loading channels and flowed into their respective chambers from the larger gaps driven by gravity and capillary action but were prevented from flowing out of the chambers from the smaller gaps. After the beads were loaded, the chip slabs were treated at 37 ºC for 30 min to remove liquid within the chambers via evaporation. This treatment caused the beads to remain in the chambers when the slab with the chambers was peeled off from the slab with the loading channels. The slab with the bead-loading channels was removed, and another slab with a single channel was aligned over the bead array to generate weir microstructures with the beads confined in the designated positions. (B) Fluorescent images of the microbeads confined in the microfluidic channels after DNA SNP genotyping by a primer extension reaction. Reproduced from [77].

Microfluidic technology is also often exploited for producing spatially defined droplet arrays [84] via compartmentalization of liquids and enzymatic reaction mixtures. This is used in couple with limiting dilution technique where a sample of interest is diluted and distributed into multiple compartments in a way when the each compartment contains one or less target nucleic acid molecule. Following amplification and counting of number of compartments showed positive amplification result reveals the initial number of the targeted molecules in the sample allowing quantitative analysis of ultimate precision and accuracy [85]. This compartmentalization can be realized in form of water in oil emulsion [86, 87] where each water droplet is considered as unique and independent reaction volume; or by distribution of the reaction mixture across
hundreds and thousands of individual microfluidic vessels [88–90]. The approach was successfully shown both for PCR [87, 88, 91, 92] and isothermal amplification techniques (LAMP) [89]. Zhang et al [90] showed implementation of the microfluidic arrays full cycle of nucleic acids analysis including DNA/RNA extraction and their real-time amplification (Fig. 10).

**Fig. 10** Schematic of the DNA/RNA extraction and following quantitative amplification using microfluidic spatially defined droplet arrays. (a) An aqueous phase containing DNA, RNA, and proteins was isolated in the microwells. (b) Organic phase consisted of phenol, chloroform and isoamyl alcohol was introduced into the headspace channel with continuous forward and reverse flow. The nucleic acid purification chip was inverted in this step as the organic phase has greater density than the aqueous phase. (c) Proteins were transferred from the aqueous phase into the organic phase, while DNA/RNA was retained in the aqueous phase. (d) The organic phase was expelled from the headspace channel and evaporated under vacuum while nucleic acids in the microwell were concurrently dried (e, f). Residual organic phase was further decontaminated by repetitive washing with 70% ethanol and vacuum evaporating. (g) q-RT-PCR reaction mixture was loaded into the microwells. (h) Microwells were covered with mineral oil followed by on-chip q-RT-PCR amplification. Reproduced from [90].
Amplification on hydrophobic-hydrophilic microarray patterns

The creation of gradients and patterns with different hydrophobic-hydrophilic properties is an indirect way of separating multiple reacting mixtures. In this case covalent tethering of PCR primers or molecular probes becomes optional since mass transfer and cross-talk between adjustment reaction mixtures is fully prevented.

Amplification of hydrophobic-hydrophilic patterned surfaces has been demonstrated by Li et al [41]. In their work they used a hydrophobic-hydrophilic patterned chip with physically isolated primer pairs on the hydrophilic spots to perform highly multiplexed PCR. In the method specific primer pairs were dispensed onto the hydrophilic spots of the microarray and the array was then covered with a PCR mixture containing all reagents except the primers. This enabled multiple unique PCR reactions to proceed simultaneously and independently.

Another approach utilizing hydrophobic-hydrophilic arrays with no immobilized primer has been shown by Shi and co-workers [28]. The authors made a pattern of hydrophilic spots on a glass surface and submerged it under mineral oil. Subsequently multiple PCR mixtures were introduced under the oil in such way that the mixtures adhered to the hydrophilic spots (Fig. 11). The design of the hydrophilic patterns was created in such a way that the locations of the adhered droplets were aligned with the optical system of the conventional real-time PCR thermo-cycler. This was then used for real-time kinetic monitoring of the PCR amplification.

**Fig. 11** Chip fabrication process for performing real-time single bacterial cell PCR on an array of adhering droplets. A stencil made of a 2 mm thick PDMS sheet was adhered on a cover slip (a). The assembly was placed in a plasma cleaner for plasma exposure (b). After plasma treatment the stencil was removed, leaving an array of hydrophilic circular areas on a more hydrophobic background (c). A PDMS frame was placed to surround the hydrophilic array to confine a pool of oil (d). Mineral oil was loaded inside the PDMS frame (e). Finally 5 µL droplets of PCR mixture were pipetted onto each hydrophilic area (f). Reproduced from [28] with permission from The Royal Society of Chemistry.
New approaches for real-time monitoring of solid-phase PCR kinetics

One of the most useful techniques to monitor the kinetics of solid-phase nucleic acid amplification has been based on fluorescent detection. Methods have included the use of both fluorescently labelled DNA probes and primers as reporter molecules [30], as well as non-specific fluorescent dyes [23]. These approaches possess both superior sensitivity and specificity of detection however require sophisticated optical systems with different band pass filters. As a result new, simple and robust detection methods are being investigated.

Surface plasmon resonance spectroscopy

A very promising approach has been demonstrated by Pollet et al [29]. They have shown, for the first time, a new tool for real-time monitoring of solid-phase PCR kinetics using surface plasmon resonance (SPR) with gold-nanobead signal enhancement. For this a forward primer was immobilized onto a fibre optic sensor (FO-SPR) surface while a reverse “liquid-phase” primer was linked to a 15 nm gold nanoparticle for signal enhancement (Fig. 12). Successful surface amplification led to the increased concentration of gold nanoparticles attached to the surface which resulted in a subsequent shift in the SPR wavelength. The specificity of the amplification was confirmed by melting temperature analysis which gave a very sharp and narrow melting point peak. An advantage of the SPR-assisted detection system is the ability to detect amplification products in non-transparent media which, for example, could be potentially useful in monitoring direct PCR amplification from blood [93, 94]. A similar SPR detection technique has been utilized for isothermal amplification kinetic monitoring [74, 95, 96].

![Experimental design of FO-SPR PCR cycling](image)

**Fig. 12** (A) Experimental design of FO-SPR PCR cycling: a) DNA template, b) forward-primer-functionalized FO-SPR sensor and c) reverse-primer-functionalized Au nanoparticles. 1–3) Double solid-phase amplification
process, resulting in the synthesis of an amplicon on the surface of the SPR sensor during extension and a subsequent shift of the SPR wavelength. and (B) Shift of the surface plasmon dip during continuous PCR cycling and DNA synthesis on the sensor surface. Presented as the SPR wavelength change relative to the 50 °C baseline (665.5 nm). Insert: plot of the threshold Ct versus the logarithm of concentration for a signal threshold of 2 nm. Reproduced from [29].

**Electrochemical detection**

Electrochemical monitoring of DNA-DNA interactions on gold [97–99] or indium tin oxide (ITO) [100] surfaces has also been attracting attention due to its simplicity of detector integration into a microarray platform. Won et al [97] demonstrated electrochemical real-time PCR on a fabricated gold electrode-patterned glass microarray using *Chlamydia trachomatis* DNA as a target gene. Kinetic PCR monitoring in this system was based on a decrease in the apparent diffusion rate of methylene blue dye after binding with amplified DNA. The system showed a quantitative analytical response over a broad range of initial DNA copy numbers.

An unusual use of the well-known anticancer drug doxorubicin as an electrochemical label was shown by Lu et al [101]. The authors used doxorubicin for electrochemical detection of epididymis-specific protein 4 (HE4, serum biomarker for ovarian cancer) via isothermal rolling circle amplification with a surface immobilised primer. A linear range in concentrations between 3 – 300 pM, with a detection limit of 0.6 pM, was successfully achieved. A combination of this approach with amplification on hydrophobic/hydrophilic patterns [28, 41] may potentially push forward the development of low-cost point-of-care microarray-based diagnostics.

**Conclusion**

The development of highly sensitive, specific and multi-parametric methods for the detection and analysis of nucleic acid is of paramount importance for fundamental life sciences and DNA diagnostics. This is especially true in light of increasing risk of epidemic outbreaks, bioterrorism and genetically induced diseases. These methods are expected to provide timely and efficient nucleic acid analysis and diagnosis that are cheaper, faster and more sensitive. Consequently, the merging of enzymatic reactions, which provide high sensitivity along with ultimate specificity, with microarray technologies that have multi-parametric analysis capabilities appears to answer this challenge. To this end the emergence of “active” arrays may provide the required sensitivity and selectivity for nucleic acid identification and genotyping. A large variety of enzymatic reactions have been shown to be successfully performed on microarrays. This has mainly comprised of classical examples of PCR
amplification and its variations. While progress has been made in implementation of these techniques on classic planar surfaces, as was proposed at the advent of microarray technologies, it is quite clear that the technology is being pushed to be transferred to a microbead-based platform. This in turn is explained by the higher surface-to-volume ratio of microbeads which results in better mass and heat transfer between active microarray spots and the surrounding media. Immobilization of oligonucleotides (amplification primers) on the microbead nanostructured surfaces also helps control spatial separation between the primers. This not only simplifies stERICALLY hindered surface hybridization of the target nucleic acids but, even more importantly, facilitates the binding of the enzyme to the hybridized complex. As a result of these efforts there has been a boom in the field of next generation sequencing technologies which employ solid-phase PCR as the main tool for both generating immobilized monoclonal templates and solid-phase sequencing reactions.

However, in order to provide highly specific PCR-based amplification of multiple different targets on a solid-phase fast cooling/heating and precise control of temperature regimes are essential. This affects the complexity of the experimental setup and often makes it unsuitable for the use as a platform for the development of point-of-care applications. To address this challenge, within the last few years, there has been a shift towards the use of nucleic acid amplification techniques based on isothermal amplification such as rolling circle amplification (RCA). This very robust technique possesses ultimate sensitivity as low as a single DNA molecule and can be easily implemented for detecting not only nucleic acids but proteins, small organic molecule and even inorganic ions. However, single-stranded DNA has to be used as a target for these kinds of applications, which is not always a case for real-life molecular diagnostics. New isothermal amplification techniques, borrowing naturally existing multi-enzyme isothermal mechanisms of nucleic acid turnaround, such as helicase dependent amplification (HDA) and recombinase assisted amplification will potentially overcome these limitations.

Finally, the integration of these systems into microfluidic-based microarrays, accompanied by sensitive label-free detection systems such as surface plasmon resonance spectroscopy and/or electrochemical detection, potentially holds great promise for the qualitative and quantitative development of multi-parametric in-field and personalized point-of-care diagnostics.

References


