

Introduction

Array Technology—An Overview

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Summary

Microarray technology has its roots in high-throughput parallel synthesis of biomacromolecules, combined with combinatorial science. In principle, the preparation of arrays can be performed either by *in situ* synthesis of biomacromolecules on solid substrates or by spotting of *ex situ* synthesized biomacromolecules onto the substrate surface. The application of microarrays includes spatial addressing with target (macro) molecules and screening for interactions between immobilized probe and target. The screening is simplified by the microarray format, which features a known structure of every immobilized library element. The area of nucleic acid arrays is best developed, because such arrays are allowed to follow the biosynthetic pathway from genes to proteins, and because nucleic acid hybridization is a most straightforward screening tool. Applications to genomics, transcriptomics, proteomics, and glycomics are currently in the foreground of interest; in this postgenomic phase they are allowed to gain new insights into the molecular basis of cellular processes and the development of disease.

Key Words: Applications; array technology; cell and tissue arrays; combinatorial science; DNA arrays; overview; roots; potential and problems; preparation; protein arrays; saccharide arrays.

1. Introduction

Around the turn of the century molecular biology has moved toward new frontiers. The sequencing of the human genome allows to describe a complete picture of the genetic heritage of humans and the same is true for many other organisms. This, in turn, allows medicine to become, at least partly, a molecular science. In this primary state of development, the sequencing efforts yield a host of data. The interpretation of these data will require a long time and a huge effort. As it turns out, the cells, tissues, or even whole organs are an ever more complex network of biological interactions. How long will it take—or will it ever be possible—to

understand more than the main mechanisms, which allows the organism to respond to the diverse internal and external signals and stimuli (1)?

When science takes a leap forward, this is generally also connected with the development of new technologies. Molecular medicine owes much of its impetus to combinatorial science. In the area of applied combinatorial science, arrays are perhaps the most popular technology. In the early years, when combinatorial chemistry was promoted by peptide chemists, the array technology was rapidly adopted by nucleic acid chemists, who developed arraying of nucleic acid sequences as a tool for genomic research. Today, the interest turns to more distant goals: protein arrays are in focus, arrays of whole cells or of tissue samples have started to be investigated.

With each generation of new arrays one can gain insight into more and more complex biological processes and their behavior in the “normal” and “pathological” state, which means into the development of disease. Arrays of oligonucleotides are now almost in routine use for the detection of mutations or other aberrations of the genome. From libraries of cDNA, one could learn to read out and compare the transcriptome, which in turn, is related to the proteome. More information about occurrence and function of proteins is further furnished by protein libraries. The step from the genome and the proteome to the “phenome” might take a longer time. Now, scientists are also investigating on the glycome and other “-omes” might lie ahead.

Of course, array techniques are not confined to molecular biology and molecular medicine. The development of new drugs has been accelerated by orders of magnitude through libraries of organic substances (2) and materials science also profits from these new developments (3).

1.1. Definitions

At this point it might be necessary to introduce some definitions: arrays are subspecies of combinatorial libraries. In general, a combinatorial library of substances is an arrangement of molecules of closely related and systematically varied structure. Thus, an oligonucleotide or peptide library might contain all sequences of a given length, or a defined subset of sequences. In another case, for materials' research it might be interesting to look at a macromolecular scaffold substituted with groups systematically varied according to their electronic nature and many other variations are conceivable.

The different species, which sum up to a library, can be called “elements.” Thus, a library of nucleotide triplets would contain $4^3 = 64$ elements, a complete library of tripeptides made up of 20 genetically programmed amino acids would count $20^3 = 8000$ elements. The rationale behind constructing and using libraries is the possibility of subjecting all elements or a subset of individual

elements at the same time, to the same kind of reaction or interaction with an analyte molecule. The information gained in this manner might lead to the elucidation of the analyte's sequence or structure, or in other cases, may identify lead structures for pharmaceutical development, to mention just two out of many possible applications.

There are different formats for combinatorial libraries. The simplest would be a solution containing a random number of differently structured molecules, for example, mixed sequences resulting from a random oligonucleotide (4,5) or peptide synthesis. Such a procedure might be valuable in certain cases, for example, in the generation of "aptamers;" however, the different elements are prepared as a mixture. The elements cannot be separated, i.e., spatially defined, and, thus, cannot be individually addressed.

Libraries, in which the individual elements are bound to particles (beads), are easily prepared by the ingenious "split-couple-combine" (or "split-and-mix") procedure (6–8). In such libraries, the different elements are spatially separated. Each bead contains one individual sequence. However, the locus of each sequence is not defined, as the oligonucleotide or peptide sequence bound to a given bead is, *a priori*, not known. "Deconvolution" procedures are necessary, before individual sequences/beads can be addressed.

Simplicity of application is the striking feature of arrays. Arrays are libraries, in which the different elements are bound to the surface of a "substrate," for example, a semiconductor "chip," or a plastic plate, or film. Location and structure of each element bound to the substrate is predefined. Therefore, each element can be individually addressed (9–12). Arrays can be prepared by spotting and immobilization of the ready-made elements, for example, oligonucleotide or peptide sequences. This mode might require an enormous preparative effort accompanying the preparation of the "chips." The other alternative is the "*in situ*" synthesis of the desired sequences on the surface of the substrates. This, in turn, requires specific surface chemistry and a good deal of engineering (13,14). Despite these difficulties arraying techniques are available, which allow to combine a very large number of elements on a limited space (up to some $10^5/\text{cm}^2$) (15,16). They have the potential of easy handling, for instance, in diagnostic tests. However, the higher the "density" of spots per unit square, the more complicated is the detection and evaluation of individual signals (17).

Because there appears to be some confusion in the literature, the following nomenclature is adhered: the given macromolecule (sequence), which is used to analyze an unknown macromolecule and which is, in most cases, the immobilized interaction partner, will be called the "probe" or "capture probe." The unknown interaction partner whose structure or sequence is interrogated, will be called the "target" or "analyte."

1.2. Arrays Are the Result of Interdisciplinary Efforts

Evidently, despite this seeming simplicity of application, a number of problems are connected with the preparation and application of arrays; the development of array techniques is, in turn, the fruit of an interdisciplinary effort. First of all, the design of an array requires knowledge about the reactions or interactions, which are to be performed with the different library elements. Making the arrays further involves the solution of specific preparative problems. Given the example of oligonucleotide or peptide sequences, such arrays could not be prepared without the development of massively parallel automated synthesis of such oligomers of high purity, either as accompanying technology, or as on-chip fabrication. The knowledge regarding surface science, design of highly reactive surfaces, and study of their reaction with peptide or nucleotide sequences is necessary. At this point, the chemist and biochemist must join efforts with engineers to position elements exactly and reliably on given spots and to deliver automatically either nucleotide or peptide sequences or reagents for their *in situ* synthesis. The urge for miniaturization requires the exact positioning of ever more minute quantities of often highly labile substances.

The application of array technology to molecular biology or medicine essentially requires a new type of analytical science. Crucial is the detection of reactions or interactions on an extremely small scale, essentially down to the single molecule respective the single event of interaction (18). To this end, ultrasensitive detection methods have to be developed. Most of them are based on labeling techniques (19), but more recently also on electrochemical methods (20). The application of such methods to the screening, particularly of high-density arrays, also requires engineering and instrumentation.

As stated before, the result of such screening procedures is a host of data and their validation is the first task followed by their interpretation. Here, combinatorial science has to collaborate with information technology and biomathematics (17). The final step is the interpretation, which is mainly in the hands of medical science, or pharmacology, or whatever users of the information.

1.3. Roots

Having described the interdisciplinary efforts to develop arrays, it might be adequate to retrace some of the roots of this technology. Based on the author's expertise, the discussion is confined mainly to the example of nucleic acids and their interactions, with an occasional flashlight on peptides.

Two background developments has to be mentioned, when reviewing the beginning of those lines of work, which later converged to array technology:

1. The availability of synthetic nucleic acid fragments and their immobilization to solid supports.
2. The development of nucleic acid hybridization to the point, where it could be exploited for diagnostic purposes.

Synthetic nucleic acid fragments (the term “oligonucleotide” is used for chain lengths up to 100 bases and beyond) were, in the beginning, rather exotic substances. Despite the fact that suitable protection schemes were available at an early date, the preparation of even short oligonucleotides in solution was quite laborious. The total synthesis of two genes in the laboratory of Khorana (21) was an undertaking that had the dimensions of “big science.” Although these gene syntheses were going on and were viewed with admiration, a new and much simpler methodology was being developed. Merrifield (22) and Letsinger (23,24) developed—in parallel and independent from each other—the polymer supported synthesis of peptides and oligonucleotides. Whereas, the “solid-phase” technique was an immediate success in the peptide field, polymer-supported oligonucleotide synthesis took many years to develop into routine, and finally to the automated methods that are used today.

The hybridization properties of nucleic acids have become evident, as soon as the double-stranded nature of nucleic acids was known. Yet, the application of support-bound oligonucleotides took a longer while and was closely connected to the availability of synthetic nucleic acid fragments. In 1972, Astell and Smith first described the immobilization of an oligonucleotide conventionally prepared in solution to cellulose and the use of these columns for chromatographic separation of the complementary strand, which was subsequently released by denaturation (25). Wallace et al. (26) were the first to show that synthetic oligonucleotides could be used as discriminating hybridization probes. In their studies they used sets of probes of mixed sequence (27), which corresponds to solution-phase libraries (*see Subheading 1.1.*). The notable feature of these probes was that for the first time they allowed to distinguish between full match and a single mismatch.

During this time, the synthesis of oligonucleotides was still performed by the nucleoside phosphotriester method, which, within the following years, was almost completely replaced by the use of nucleoside phosphite synthons (28), especially the phosphoramidite chemistry (29). This new chemistry and in particular its adaptation to mechanized synthesis, was undoubtedly the breakthrough regarding the application of synthetic nucleic acid fragments (30). With a rapidly growing demand for synthetic oligonucleotides, mainly for use as probes and gene fragments, improvements to make the existing methodology faster and more efficient were necessary. The need was felt to prepare more than one oligonucleotide in a single run, which was, for example, realized manually by using a battery of syringes (31,32). Yet, the performance of many parallel operations was still tedious and with the risk of error. Thus, the idea of using segmental supports for multiplexed oligonucleotide synthesis (33) was a definite step forward. This technique was adapted to semiautomated (34,35) synthesis and somewhat later, a prototype of a machine for fully automated parallel preparations of multiple oligonucleotide fragments was described (36).

Today the engineering of multiplexed oligonucleotide synthesis has been improved to a point, where we talk of “high-throughput” synthesis. Every year new machines are being constructed, which allow the parallel preparation of ever increasing numbers of oligonucleotides. In a recent report 1536-well microplates were used to synthesize, in parallel, a corresponding number of oligonucleotides in about 10 h (37), and more record-breaking news is probably in store. The result of these efforts is a dramatic decrease of cost for standard oligonucleotide sequences. Therefore, procedures requiring large numbers of prefabricated probes or primers, for example, spotting of arrays or cDNA preparation, can now be handled with much less expenditure.

1.4. Combinatorial Chemistry: The Beginning

Combinatorial chemistry results from multiplexed synthesis with the aim of screening individual elements for a given activity or interaction. Unlike the synthesis on segmental supports described before, the products of multiplexed synthesis now are supposed to remain bound to a support particle or to a support surface for the purpose of interaction and screening. The two lines have the same roots, but combinatorial chemistry deviated in the 1980s, when sets of peptides were synthesized by a multiplex technique with the aim of establishing structure–activity relationships (38,39). The seminal idea probably was born in the research group of Geysen (40), who prepared sets of peptides in small quantities, but large numbers on the heads of polyacrylate pins. The pins were arranged to a kind of brush; the pinheads could be dipped into multiwell plates, where each well would contain the individual reagents for chain elongation.

A parallel development was initiated by Furka (6–8): the so-called “split-couple-combine” (“split-and-mix”) method. The essence of this method, which was also originally developed in the peptide field, is that before every coupling cycle the amount of polymer support is equally divided into a given number of batches. To each batch is coupled a different amino acid. After finishing this cycle the different batches are mixed and then divided again into as many portions as amino acids are to be added. This coupling in split batches, combining and splitting again for the next coupling is continued until the combinatorial synthesis is completed. The feature of this method is that, in the end, each bead contains a single individual sequence, which could be used, for instance, for studying epitopes with affinity toward monoclonal antibodies (41). However, the drawback of this method is that the sequence finally attached to the bead is, *a priori*, not known. This involves an identification procedure off single beads, a problem, that was first solved in the case of the peptide libraries (8,41), and only much later for the case of oligonucleotide libraries (42,43). Generally, it might be because of this

“deconvolution” problem that the interest in beaded libraries has by far not matched the interest in arrays, to which we will now come back in the brief survey.

2. Array Technology Picks Up Speed: Masks or No Masks

In the beginning, there was some irritation among scientists that they should use wild mixtures of millions of compounds for the identification of lead structures instead of pure and well-characterized substances. But it was quickly recognized that “irrational drug design” (44) would speed up the identification of drug leads by orders of magnitude. Thus, since the early 1990s a fast development ensued, resulting in a nearly exponential growth of the number of array-based publications (47) (in the meantime probably >10,000) and a comparable number of patents (45). In view of this enormous amount of publications and a number of recent books (13,14,46–53), this introductory chapter does not attempt a complete coverage of the information available, not even of parts of array technology. Rather, an attempt is made to look at the background and beginnings of array technology, whereas, the second part that describes the recent progress and future projections is to a large extent, a “review-of-reviews” with links to important and helpful summaries and compilations.

In the first moment, if one had to concentrate first on DNA arrays and, here, on technology-oriented publications, it is difficult, to find a way through the numerous intertwined lines of development. A clear distinction is between *in situ* synthesis of oligonucleotides on substrate surfaces and the deposition (“spotting”) of ready-made sequences, here again, between chemosynthetic oligonucleotides and enzymatically transcribed DNA (cDNA).

For *in situ* synthesis it is perhaps possible to distinguish three main tracks: (1) the use of masks with microfluidic devices; (2) addressing through light-directed deprotection; (3) maskless technologies, and also crossover developments occurring between (1), (2), and (3). The main steps of *in situ* synthesis of arrays are described in **Fig. 1A**.

2.1. In Situ Synthesis Involving Masks

The seminal papers in this area are a result of the work of Southern and his colleagues (54–59). Arraying was done by masks, which create a system of microfluidic channels on the surface of a glass substrate, through which the reagents are delivered from a DNA synthesizer. By dislocation of the masks a “grid” of different oligonucleotides is prepared, which, by suitable chemistry, remain bound to the glass surface. Another research group used polypropylene substrates, which could be functionalized on the surface by plasma discharge in the presence of ammonia (60). A 64-channel automated delivery system for

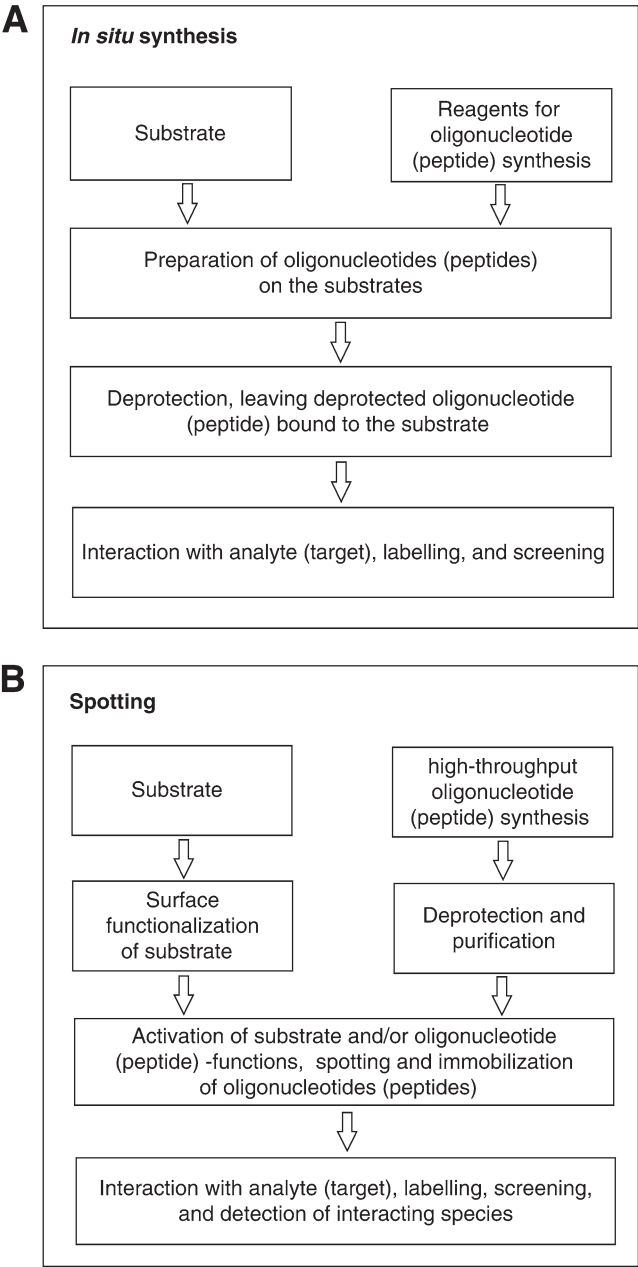


Fig. 1. Two routes to the preparation of oligonucleotide arrays.

reagents could be rotated 90° to produce a checkerboard array of 64×64 sequences (61). Full mechanization was achieved using an “endless” polypropylene tape, functionalized by “wet chemical” oxidation + reduction, which is automatically positioned above a meander-shaped “reaction chamber,” thus creating a “bar-coded” one-dimensional array of frame-shifted oligonucleotide sequences (62,63).

Although the techniques described so far all use conventional phosphoramidite chemistry for oligonucleotide chain lengthening, a parallel route of development was initiated by the work of Fodor at the Affymax Research Institute. In his seminal publication (64), Fodor described the use of photolabile protecting groups for the ends of the chains grown on the substrate surface. This chain lengthening method is based on a photolithographic process. Spatial addressing of given spots on the substrate is ensured by photolithographic masks. After photodeprotection all other steps of the synthesis cycle are performed simultaneously for all elements, but chain lengthening will occur only at the spots previously deprotected. This combination of solid-phase synthesis with photolithography, which was demonstrated for peptide preparations (64), but rapidly adapted to oligonucleotide synthesis, is particularly featured by the possibility of generating extremely high density arrays (65) approx $300,000 \text{ spots cm}^2$ and beyond (66). Crucial for the success of this method is the facile and quantitative removal of the 5'-protection, therefore, the search for new photolabile protecting groups has been greatly stimulated (67–69). Despite these efforts, the yield of the light-directed deprotection is not as close to quantitative as that of the usual acid deprotection. Therefore, modifications have been developed, in which conventional acid deprotection is gated by photolithography using semiconductor photoresistent masks (70,71). In an alternative method called micro wet-printing, structured membranes with integrated delivery system are pressurized onto the chip surface. This allows conventional phosphoramidite chemistry to be performed on given spots of $2 \mu\text{m}$ diameter with a coupling efficiency of $>98\%$ (72).

Recently, new interest arises in arrays prepared by *in situ* synthesis of oligonucleotide analogs. Especially peptide nucleic acid (PNA) (73) or locked nucleic acid (LNA) (74) sequences are interesting as capture probes, as they show tighter and more specific binding toward complementary nucleic acids than DNA itself.

2.2. Maskless Arraying Techniques

Although the techniques using masks are now routine procedures and have reached a high degree of sophistication, they have not been found fully satisfactory. For independent addressing, masks essentially have to be restructured after each synthesis cycle. This makes the preparation of custom-synthesized arrays

quite expensive. Therefore, techniques avoiding the use of masks have been promoted during recent years. Systems of computerized micromirrors have been used to direct the light for deprotection at given dots on the array, an innovation that makes the preparation of high density arrays faster and more flexible (75,76). Coupling of this technique with the use of a sensitizer has considerably improved the yields of photochemical deprotection (77). To avoid the undesired diffusion of reagents, particularly acid, the surface has been microstructured, thus creating small wells for each individual spot (71). However, this procedure reduces the resolution, leading to a spot size around 150 μm .

2.3. Jetting or Dispensing Devices

Generally, if devices for jetting or dispensing chemicals are used for *in situ* synthesis, they have to cope with three problems, namely the evaporation of solvents, access of air and moisture, and the aggressive nature of some of the reagents used during the phosphoramidite cycle. Piezoelectric dispensing devices (78) solve these questions by fitting a closed “reaction chamber” over the spot to be reacted. For protection against air and moisture, while dispensing phosphoramidites or H-phosphonates, protogene has integrated the liquid reservoir, fluid system, and piezo jetting elements into closed ceramic substrate housing up to 64-jetting units (79). Such precautions are not necessary, if acid deprotection is the only step handled by a dispensing unit. Combion handles detritylation by using $\text{ZnBr}_2/\text{CH}_2\text{Br}_2$ /isopropanol in glass capillaries surrounded by piezo elements (80). In the case of a shuttling device, the deprotection step is done by dispensing acid to predetermined spots on the array by a bubble-jet using diamond technology to avoid corrosion. For the other steps of the phosphoramidite cycle the total array is relocated over a microfluidic device, through which all reagents of the phosphoramidite cycle are delivered (81). An “inverse” masking strategy deposits a cover layer of polymer over all elements, which are not included in the following chain elongation, then proceeds normally with the synthesis cycle and then washes the polymer cover away, before starting a new chain elongation (82).

An important criterion, when using dispensing or jetting devices, is the drop size respective the quantity of liquid ejected with each droplet. Although the ejection of 10–100 pL is standard in many approaches (83), it is possible to reduce the ejected volume to <2 pL with recent techniques (81).

Quasi an “inversion” of Geysen’s old strategy (40) is the “microcontact printing” technique, in which reagents are delivered to spots on the surface containing the growing oligonucleotide chains by silicon rubber stamps (84–86).

2.4. Spotting is Different

The techniques of producing arrays by spotting of synthetic oligonucleotides or enzymatically prepared nucleic acids (cDNA) are different from *in situ* synthesis

on substrate surfaces. Here, the problems of oligonucleotide synthesis are quasi “outsourced.” Arrays are prepared from ready-made oligonucleotides or nucleic acids. The questions that have to be solved from the standpoint of chemistry, relate to the choice of a proper functionalized substrate and the immobilization of the (likewise functionalized) oligonucleotide chains to the activated substrate surface.

2.4.1. Noncovalent Linkage of Nucleic Acids to Surfaces

The possibility of immobilizing nucleic acids to membranes was already exploited in the early years of nucleic acids chemistry by Gillespie, who immobilized DNA noncovalently to a nitrocellulose surface (87). In many cases, it is sufficient for immobilization, if the polyanionic nucleic acid chain is simply bound to a positively charged surface through electrostatic interaction (88,89). Another “classical” way of noncovalent binding is through biotin–streptavidin interaction, which was introduced by Wilchek >30 yr ago (90). Noncovalent adsorption to surfaces is also the rationale behind the different blotting procedures, which transfer nucleic acids from the surface of one substrate to another (91–93).

2.4.2. Covalent Linkage of Nucleic Acids to Surfaces

For routine diagnostics and in view of multiple reuse a noncovalent attachment of the probe is usually not sufficient. Therefore, a multitude of methods for covalent anchoring of oligonucleotides to substrate surfaces have been developed. An overview of the different steps involved in spotting and covalent immobilization is given in **Fig. 1B**.

2.5. Substrates and Immobilization Techniques

Any spotting procedure starts with the choice of an appropriate substrate. For simplicity, glass (for instance in the form of microscopic slides) is by far the preferred substrate, but a variety of other polymers can optionally be used (94). The substrate surface mostly contains hydroxyl, amino, or carboxyl groups as primary functionalities. By a secondary reaction these are converted into highly activated functional groups, such as isothiocyanate, N-hydroxysuccinimide, or tresyl chloride residues. Generally, this question of functionalization leaves a wide field for the imagination of chemists (94). As a general rule, such highly activated species are more stable than in solution, when they are “isolated,” i.e., prevented from diffusion, through immobilization to the surface of a polymer. For facile attachment, the preformed oligonucleotide chains are also functionalized, generally on one of their termini. The functional groups chosen for this purpose are mostly hydroxyl, amino, or thiol groups, i.e., groups different in their reactivity from the functionality of the nucleobases. An activation of the oligonucleotide termini is generally avoided, because there would be a risk of backbiting into the oligonucleotide chain. Analogous considerations can be forwarded for the spotting of peptide

chains. Because the signal intensity can be increased by increasing the surface loading with oligonucleotide (or peptide) probes, attempts have been made to transform the planar into a structured surface (the term “three-dimensional surfaces” has been coined for this approach). This could be done by applying a porous coating to the glass plates, either with cationic or hydrophobic polymers (95), or with dendrimers (96), or with an ultrathin layer of star-shaped polyethylene glycol (97). A 100-fold increase of the “density” of functional groups on the substrate surface has been reported (95). An often used coating is also the application of a thin layer of sputtered gold onto the glass surface, which allows the attachment of thiol substituted oligonucleotides (98–100).

An alternative to the direct immobilization of libraries of oligonucleotides or peptides to nonstructured or structured substrate surfaces is to cover the surface with a layer of oligonucleotide loaded microbeads (101,102). Films of colloidal silica were applied onto flat glass surfaces (103). Extensive studies on such a “three-dimensional” surface showed, that a silica particle layer of 0.5 μm thickness resulted in a 20-fold increase of hybridization signal, although about 20% of the surface area are inaccessible to probe synthesis and >30% are inaccessible to hybridization with target.

It is a small step forward from structured substrates to the introduction of microchannels, which contain the sites for oligonucleotide growth or immobilization (104,105). Delivery of solutions through these microchannels speeds up washing and facilitates hybridization (106). Additionally, enzymes can be immobilized inside the channels to process reactants, thus, progressing from microarray to lab-on-a-chip (107).

A potential disadvantage of immobilizing oligonucleotides to substrates by chemical reactions is the necessity for removal of reactants and byproducts. This is avoided, if photochemical techniques are employed for the immobilization (108,109). If this photoreaction is selective enough, it provides for a clean and convenient attachment to the substrate.

2.6. Spotting of cDNA and Other Long Nucleic Acid Fragments

Monitoring mRNA synthesis starts with the reverse transcription of mRNA into cDNA using synthetic oligonucleotide primers. In view of the small quantity of mRNA available, the cDNA synthesis is usually followed by PCR amplification. In PCR, 5'-amino-modified primers are used, which directly allow spotting of the PCR products onto activated microarrays (110). Because amino-modified primers are more expensive than unmodified primers (generating unmodified cDNA), the spotting of such unmodified cDNA was investigated by several groups. Additionally, it was found necessary to increase the stability of the bound cDNA. For this purpose, a postimmobilization ultraviolet (UV) crosslinking can be applied (111). Alternatively, the substitution with an aromatic amine allowed

diazotization of the substrate surface. The diazonium groups could then be reacted with the nucleobases of unlabeled target DNA (the authors assume, that binding can occur to A, C, and G). This was then followed by hybridization with labeled probe (112).

Judging from the literature and from the experience (113), the mode of attachment of a DNA sequence to the substrate is not of utmost importance to the hybridization behavior. Generally, *in situ* synthesized probes are bound to the substrate through their 3'-end (or through the 5'-end; synthesis in reverse direction has been reported in refs. 114 and 115). When spotting shorter oligonucleotides, terminal attachment is generally assumed, and has been investigated through the formation of self-assembled monolayers on gold surfaces (98). In the case of longer DNA chains, especially cDNA, it is more likely, that a multipoint attachment to the substrate occurs, particularly if UV crosslinking is used for tighter binding. A comparison of hybridization behavior between 50 and 70 base oligonucleotides spotted and UV crosslinked onto aminated glass slides vs deposited PCR amplicons of cDNA did not show any significant difference in sensitivity (116). A detection limit of about 10 mRNA copies per cell was reported (117). It is hard to imagine, that in such a case a double-helix of the B-type could be formed on hybridization. The same question arises, for example, on spotting of DNA on nitrocellulose membranes, which is also a multipoint attachment. There is little mention in the literature regarding the structure that may be formed on double-strand formation. Lemeshko (118) postulates a partially unwound and significantly extended double strand, where the loss of base stacking is compensated by electrostatic interactions between DNA and substrate matrix.

3. Nucleic Acid Hybridization: Detecting the Difference

Most applications of nucleic acid arrays are based on hybridization techniques. In contrast to DNA- and RNA-hybridization in solution, which has been intensively studied, hybridization processes on surfaces are more complex and their assessment presents more difficulties. Here are some of the complications:

1. One of the interacting partners is immobilized.
2. The quantities of interacting partners are often extremely small.
3. The surface density of the immobilized probe cannot be increased indefinitely.

Furthermore, in the case of *in situ* synthesis the immobilized oligonucleotide partner is inevitably a mixture containing, in addition to probe sequence, a large number of truncated and failure sequences (for calculation of failure sequences see ref. 119). Model studies done by Landegren (120) stress the necessity to prepare immobilized probes with a yield of chain elongation as high as possible.

In the case of long DNA fragments spotted either on nitrocellulose or on activated glass slides, the hybridization kinetics was found to be dependent on the

concentration of the target solution, but also on the length of the arrayed DNA. The authors explain this result by the fact, that longer DNA needs less attachment sites and, thus, generates longer sequence lengths for hybridization. The nature of the substrate or the kind of surface was not found to be of importance (*121*). In contrast, Belosludtsev (*122*) showed that conditions under which the substrate surface is slightly positively charged, results in very fast hybridization kinetics.

Another critical parameter, as previously stated, is obviously the surface density of the immobilized probe. Using comparable target concentrations, the hybridization kinetics were investigated corresponding to probe densities between 2×10^{12} and 12×10^{12} molecules of DNA/cm² (either single- or double strand). All samples exhibited similar hybridization kinetics; however, in the experiment done with 12×10^{12} molecules/cm² the hybridization efficiency reached only 10% of the efficiency found with the lower density sample (a plateau being attained already at 0.2 h) (*123*). The authors explain this result by electrostatic repulsion between the immobilized sequences, which each require a certain space; additional thermodynamic considerations are forwarded in a subsequent article (*124*). As a consequence, microstructured substrates were recommended for better mixing of the hybridization partners (*106*).

Prerequisite to all hybridization-based investigations are efficient and highly sensitive detection methods. The efficiency of detection systems, in turn, is connected with different parameters, namely sensitivity, mismatch discrimination, spatial resolution, reproducibility, signal-to-noise ratio, and cost. Most detection methods rely on labeling of the target nucleic acid. After stringent hybridization and washing the presence of label on certain spots of the array indicates the formation of perfectly matched double strand. The conditions must be chosen so that duplexes with a single mismatch be excluded.

The detection technology depends largely on the type of label chosen. Fluorescent substituents are most widely used as labels. These require optical detection systems, most often confocal scanning devices. Recent technical advances are, for example, reported in **ref. 125**. An overview of fluorescence detection methods is given in **refs. 126** and *127*. In order to increase the signal intensity dye-loaded beads (*128*), gold nanoparticles (*129*), dendrimers (*130,131*) and a number of other labeling alternatives can be used. Quantitative fluorescence calibration for the analysis of microarrays has also been described (*132*). Labeling of the target with radioisotopes provides for highly sensitive detection (*15,133–135*); however, radiochemical methods are hazardous for routine clinical applications. Optical detection methods, which avoid the extra step of target labeling are, for instance, fluorescent staining (*136*), surface plasmon resonance imaging (*137*), or mass spectrometry (*138*). Heller and colleagues have drawn attention to the fact that in the usual “passive” microarray approach all spots of capture probes are simultaneously exposed to the same

hybridization conditions. The stringency of mismatch discrimination might suffer, if length, G–C content, or potential secondary structures of the probes are inadequate. Therefore, the use of an electric field is recommended to direct nucleic acid hybridization on microchips (139).

Much attention has recently been drawn to the electrochemical detection of hybridization. Often it is still a label, for example, a redox-active substituent, whose change in redox potential on hybridization is measured (140,141). A more elegant way would be to detect minute changes, for example, in impedance or capacitance, which occur on hybridization of unlabeled probe and target (142,143). With enhanced specificity and increased sophistication of electrochemical-biosensor technology, future development might even be directed toward avoiding PCR on monitoring gene expression by cDNA spotting (144).

A major concern in the application of DNA microarrays is the reproducibility and reliability of the hybridization results. Sterrenburg et al. (145) recommend the cohybridization of a common reference on all arrays containing spotted cDNA PCR products; a similar procedure was proposed by Hu (146). A system for automatic quantification of hybridization signals from cDNA arrays has been described (147). The validation of analysis results is closely connected to data handling and processing (see **Subheading 6.**). Chuaqui (148) proposes a dual strategy: *in silico* comparison with previous information on the one hand, and comparison with other experimental methods on the other hand (see also **ref. 149**).

4. Applications of DNA Chip Technology: Business Opportunities Drive Science

There might not be a reason for writing a review, or even editing a book, on array technology, if there was not such an enormous potential of applications. DNA array technology has, since long, outgrown the laboratory and entered the realm of business (40 million US \$/yr is a current estimate with a 10-fold growth predicted up to the end of this decade [150]). Concomitantly, most of the new developments since the late 1980s have been submitted for patents; some of the resulting patent battles have been spotlighted by Gabig (101). It is hardly possible in this introductory chapter to do more than highlight some of the most interesting applications, which are anyway the topic of following sections of this book. A huge volume of literature has accumulated over the last years. Most of the reviews and many articles cited in this chapter contain aspects of application in addition to novel procedures. Two recent surveys of applications, one in an article by Heller (151), another in a review by McGall (66) are mentioned.

Array technology can be used to assess essentially the complete pathway of biological information processing. DNA arrays can interrogate the composition

of genomes and find sequence aberrations (sequencing by hybridization, detection of mutations, and single nucleotide polymorphisms). By far the most effort is currently spent on the analysis of gene expression. Profiling is usually done in a differential way, i.e., by comparing the expression level of a disease-related gene to the level of expression in the healthy state. This is mostly done by two-color fluorescent labeling. An excellent introduction to microarray profiling and its potential for the study of disease was given earlier by Khan (152). In the meantime, a wide range of diseases, as well as problems in medical research and related areas, is being studied by array technology. Just to quote a few very recent publications: research is being done in the field of autoimmunity (153), parasitology (154), toxicology (155), occupational health (156), microbial forensics (157), and military medical science (158). A wide and most interesting field of current applications is cancer research. Studies on the differential expression of cancer-related genes have yielded a much more diversified picture of cancer and might lead to the development of a defined and perhaps more individual therapy (159). Printing out the “molecular portrait of cancer” (160) and taking advantage of progress in tumor immunology (161) might lead to new routes of cancer treatment (162). Also in the spotlight of recent reviews is melanoma research (163) or genetic disorders (164–166).

5. Microarrays Beyond DNA

5.1. Protein Microarrays

Although DNA arrays are extremely helpful in detecting the status of the transcriptome in a diseased against healthy state, this does not necessarily represent the status of the proteome, i.e., the ensemble of proteins resulting from the expression of a group of different genes. This is because mRNA and protein levels are often not correlated and many proteins are posttranslationally modified (167). Therefore, considerable effort is now concentrated on constructing arrays, which might serve to analyze the protein status in a given sample (168). The first question in the case of protein analysis concerns the choice of capturing probes. Often, specific antibodies or antibody fragments are arrayed, but also nonpeptidic “aptamers” or specific ligands can be used for immobilization (169). Methods of detection and quantification include, for example, fluorescence tags (170) or mass-spectrometric analysis (171), often parallel to detection methods for DNA arrays, for example, in the application of microfluidic devices. Peptides or proteins can likewise be used as immobilized ligands. In addition to the conventional methods of *in situ* synthesis (172) or spotting (173) specific techniques, such as electrospraying (174) or mass-spectrometric deposition (175) have been developed. “Reverse-phase protein microarrays” are often obtained by spotting representative protein fractions from cells or tissues. By interaction with disease-specific markers information is obtained,

which can be used in a prognostic sense or in order to design an individual therapy (176).

5.2. Saccharide Microarrays

Saccharide microarrays are the subject of very recent research activities. Many proteins, especially mammalian proteins, are substituted with oligosaccharide chains. Likewise, cell surfaces are “decorated” with glycans, mainly in the form of glycoconjugates (glycoproteins or glycolipids). These glycoconjugates have important functions within the cell: they are recognition markers for cell-to-cell communication, they promote cell adhesion, development, differentiation, and immune response (177,178), and they are also involved in disease processes, such as inflammation, tumor metastasis, or bacterial infections. Even though nature uses only a limited set of sugars and their aminated analogs as monomers, the fact that these can be linked together in many different ways, can be differently substituted, and the possibility of anomerization at several chiral centers, create, in the sum, nearly unlimited possibilities of structural variation. Thus, the information content in relatively short oligoglycans might by far exceed the possibility of information storage in oligonucleotides of similar length (179). Such considerations have opened the scene for a newly emerging science: glycomics. The task of glycomics is the investigation on the specific “inventory” of glycans, glycoproteins, glycolipids, and other glycoconjugates of a given organism, as well as the elucidation of genes and gene products responsible for their metabolism (179).

What is now called glycomics, has long been a rather neglected field of science. Similar to nucleic acids and proteins (*see Subheading 1.3.*), technological advances have now paved the way (180). On the one hand, modern analytical methods, like two-dimensional nuclear magnetic resonance or mass-spectrometric techniques, facilitate the elucidation of glycan structures, although purification and structural analysis still remain a formidable task. On the other hand, the construction of an automated carbohydrate synthesizer (181) has made it possible to prepare saccharides of given structure with a reasonable amount of time and effort (182). New enzymatic methods of saccharide synthesis (183) are an additional preparative tool.

This, in turn, has been the starting point for combinatorial chemistry in the field of carbohydrates and glycans. Combinatorial carbohydrate research can help to elucidate all kinds of interactions of carbohydrates with carbohydrates, nucleic acids, proteins, and other biomolecules (184). For these studies, a variety of specifically fluorescence-labeled saccharides are now available, in addition to the established “toolbox” of other labeled biomolecules. In addition to fluorescent labels quantum-dot conjugates, affinity and magnetic particle tagging, as well as surface plasmon resonance have been used to probe for carbohydrate interactions (184).

From this basis the preparation of carbohydrate microarrays has made rapid progress during the last years. In contrast to nucleic acid and protein arrays, *in situ* synthesis is here a rather exceptional case (185), but might become interesting with further development of solid-phase preparation methods. In addition to chemical methods, enzymatic transformations of carbohydrates bound to the surface of microarrays have been reported (186). On the other hand, numerous methods for the immobilization of ready-made oligosaccharides and their conjugates have been developed and high-density microarrays have been constructed (for recent review see ref. 177). Among the currently available methods, noncovalent linkage of unmodified carbohydrates to, for instance, nitrocellulose-coated glass slides is the simplest (187–189), but has also the disadvantage, that desorption can occur on repeated use. Tighter binding occurs with neoglycolipids (lipid-bound oligosaccharides) (177), which give rise to the assumption, that this adsorption is mediated by hydrophobic interactions. However, the trend goes definitely toward covalent immobilization of carbohydrates. In order to effect stable and region-specific immobilization of glycosides (mostly at the reducing end), chemical modification is often necessary for both the substrate and the carbohydrate. A variety of substitution and immobilization methods have been developed using, for example, the attachment of maleimide-linked carbohydrates to sulfhydryl-coated glass slides (190), of aminophenyl-substituted glycosides to slides coated with cyanuric chloride (191), or of glycosides modified with azide groups with alkynylated surfaces (192). Other alternatives are Diels–Alder (193) or Staudinger (194) ligation. Michael addition of glycosides modified with maleimide groups to thiol-derivatized glass slides also provides for stable attachment (195).

Among applications of carbohydrate microarrays protein recognition is the most prominent topic. Several studies have proven the specificity of oligosaccharide microarrays for binding carbohydrate-recognizing proteins, such as antibodies (196) or lectins (197). Lectin-carbohydrate binding affinities could be quantitatively assessed by determining IC₅₀ values for soluble carbohydrates against the carbohydrate arrays (198). By immobilizing a series of closely related structural determinants of glycans decorating the viral-surface envelope of HIV, the binding capacity of a series of gp120 binding proteins was investigated (199). In view of antibiotic resistance acquired by bacteria through transfer of plasmid DNA or overexpression of endogenous enzymes modifying aminoglycoside antibiotics, the high-throughput search for new glycosides, that bind strongly to therapeutic targets, but show little affinity to resistance- and toxicity-causing proteins, is of great importance (200–203).

Recent work by Seeberger has taken carbohydrate arrays one step further toward biomolecular diagnostics and therapy. Starting from the idea, that microarrays present carbohydrate ligands in a manner that mimics interactions at

cell–cell interfaces, such arrays could be used to study carbohydrate-binding specificities of bacteria. Because only picomols of ligand are required for such studies and microarrays can harbor a large number of different structures, such investigations allow high-throughput screening for antiadhesion therapeutics (204).

Some of the problems encountered in the preparation and use of carbohydrate microarrays have been summarized by Mellet et al. (205). First of all, they recommend covalent linkage of oligosaccharides to the chip surface, because noncovalent attachment is not very stable in the case of shorter saccharides. If possible, this should be coupled with the formation of selfassembled monolayers (see ref. 193). This covalent immobilization, however, must (1) be highly efficient and (2) be done in such a way that the carbohydrate-containing macromolecules retain their recognition properties. The latter requirement is not trivial in view of the fact, that the immobilized ligands are generally very small compared with the proteins they are supposed to bind (206).

Despite these potential restraints carbohydrate array technology is on a promising way. There is potential, for instance, for rapid diagnosis of infectious diseases, for detection of migrating tumor cells, for profiling of carbohydrate-binding proteins, and for identification of novel inhibitors of glycan–protein interactions (205). Thus, carbohydrate arrays in the future might significantly contribute to biomolecular diagnostics and therapy.

5.3. Cell and Tissue Microarrays

Very recently, cell and tissue microarrays have come into the focus of research. Using very careful methods of deposition, small numbers of cells, or even single cells, can be spotted as library elements. This can be done by structuring the substrate surface (207), by depositing cells in nanocraters (208) or in microchambers (209), in order to prevent cross-contamination. A small number of cells (eventually a single cell [209]) could be immobilized within these nanostructured arrays. Standard microcontact printing methods were employed for spotting the cell suspension. Joos et al. (210) described another method of immobilization in which poly-L-lysine-coated glass slides was used as substrates for ionostatic fixation of the cells. After depositing the cells a hydrophobic pen was used to frame around the spots. With an average of 18.3 cells per spot 400 colonies could be arrayed. In all these cases the cells remained in a viable state after spotting. Self-assembled monolayers of cysteine-terminated oligopeptides on gold-coated substrates were used to immobilize *Escherichia coli* cells. These cells showed decrease in viability on addition of phenol, exemplifying the potential use of cell chips to detect environmental pollutants (211). Some applications: in ref. 209, the authors describe the stimulation of deposited B cells with antigen, along with detection of their status before and after stimulation. Furthermore, the cells could be retrieved from the microchambers for analysis

of antibody-specific DNA. The studies described in **ref. 210** use antibodies to proteins displayed on the cell surface to monitor, in high-throughput format, specific binding to different cell types applying appropriate fluorescent-labeled detection molecules (**210**). The monitoring of RNA interference-mediated loss of function was the topic of another publication (**212**).

A considerable amount of research was triggered by a seminal paper by Ziauddin (**213**). Their method of arraying differs from the earlier-described methods, in that they deposit cells not directly, but, in first instance, spot drops of cDNA-expressing plasmid, suspended in aqueous gelatin solution. To this is added a lipid-transfection agent, and finally the whole is overlaid with a cluster of cells. By “reverse transfection” the cells pick up the plasmid and thus, start to actively overexpress a defined gene product (**214**). In this way, such “indirect” arraying of cells constitutes, essentially, a new route to proteomics (**215**). Sabatini et al. (**216**) have demonstrated two potential applications of this technique: (1) screening for gene products involved in biological processes of pharmaceutical relevance, and (2) the high-throughput screening for drug leads directly in the cellular environment. Other groups have used this technique to analyze the binding affinities of membrane-displayed antibodies (**217**) and to quantitatively assess the efficiency of plasmid DNA and siRNA transfection (**218**).

Tissue microarrays can be used for histochemical or immunohistochemical staining or for *in situ* hybridization (**219**). In this aspect they complement DNA or protein chips in diagnostics and prognostics (**220–222**). Several 10 or 100 specimens of tissue are arranged onto slides, constructed from paraffin-embedded or frozen tissue (**223**). It is envisaged, that tissue microarrays might bring about a revolution in pathology and quickly become an integral part of clinical routine. However, there is a warning that, because of the small diameter, spotted samples might not be representative for the tissue as a whole (**220,223**). This can especially be the case in heterogeneous tissues, such as tumors. In this case multiple samples must be sampled and screened, guided by experience.

6. Managing the “Data Deluge”

Being primarily a means of high-throughput diagnostics, array technologies create, first of all and above all, an enormous amount of data known as “Data Deluge” (**224**). Especially for the processing of data from gene expression monitoring, a number of algorithms have been proposed. In addition to forwarding such algorithms for high-density oligonucleotide arrays, Zhou (**225**) provides a critical review of the literature up to 2003. Links to information processing and management are contained in the review by Khan (**152**). Gene expression profiling coupled to DNA computing has been proposed (**226–228**). Baldi (**229**) developed a Bayesian framework for the analysis of microarray expression data. More on the practical side are algorithms for filtering noise from data sets

(230), for increasing the reproducibility (231) or for selecting probes with optimized uniform hybridization properties (232).

7. Array Technology: To Where Will it Proceed?

Array technology and combinatorial science in general, will certainly be one of the key technologies of this century. No wonder, that a certain amount of euphoria is expressed in many publications and reviews (e.g., refs. 233 and 234). On the other hand, microarrays are not yet a common tool in the hands of non-specialists, especially in molecular biology laboratories (12) and in clinical routine (152). So, there is still a demand for progress and improvements in many directions. New developments are also owing in the field of technology. This concerns, in particular, the quality control of the chips and the reliability and reproducibility of the results (235). Utmost care should be taken in the validation of the array data, especially if these are the basis for clinical diagnostics or prognostics (236). Furthermore algorithms will be necessary for this validation. Above all, progress must be made in the interpretation of the data collected from the application of arrays. New sequencing techniques promise to analyze whole genomes in a matter of months (151,237). But much more time and effort is needed, in order to perfect expression profiling and to correlate expression profiles to the healthy or diseased status of the organism. A wide field of technologies and applications, awaiting further development, lies beyond arraying DNA. For instance, micro-RNA, a new genetic element, which obviously plays a role in silencing of mRNA translation and in the control of cell and tissue differentiation, is just coming into the focus of research and development (151). Proteome analysis is on the agenda. Already the view goes beyond cDNA and protein arrays to arrays of tissues and cells. Novel techniques not only allow gene expression profiling in an array of spots containing small numbers each of deposited cells, or even single cells as elements (238). In the longer run viable cells spotted on appropriate grids may be used as biological “labs-on-chips.” From all these research efforts, much more has to be learned about the factors and parameters, which, in many ways, control gene expression. The ensemble of gene products, in turn, regulates the behavior of cells, and the crosstalk of cells the biological properties of tissues. Thus, chip technology may provide an “array of insights” (239) into the complex mechanisms active within biological systems (240,241).

Beyond the gain of knowledge and insights for science, what is the benefit for society, where are the warnings? Medicine expects as a major benefit the development of more and more individualized therapeutical approaches (242). This is expected to reduce side effects of current treatments or medication and to cut down the soaring cost of healthcare. On the other hand, ethical questions arise and will have to be solved by the world community. For instance: how can individuals

escape stigmatization, if molecular diagnostics reveal the predisposition to a genetic malfunction or a severe disease (243)? Clearly, there is, in this controversial situation, a responsibility of science and medicine, but above all there is a demand for further research and development, since, as described by Neumaier (243), “the furtherment of analytical/technical skills and diagnostic/medical knowledge, and quality management, are without doubt, a major integral element of ethical behavior or activity in the field of genetic diagnostics.”

8. Some Helpful Links

Here are some links, which the reader can find in the more recent literature. Companies are listed with their activities, location and websites, status 2005, in a review by Gershon (244). A list of producers of microarrays, biochips and lab-on-chips, status 2002, is given by Heller (151). A list of different equipment for the construction of arrays (status 1999) can be found in an article by Bowtell (245). Gwynne also gives a list of resources for microarray analysis (1999) (246). Scanners for microarray analysis are compiled in (247). Useful websites on applications of arrays are contained in a new review by Chaudhury (17) (2005) and also in two previous reviews (152,234). Gerhold et al. (234) also list chip formats marketed by different companies. Wheeler and colleagues (248) have compiled a list of data resources, which can be used in addition to GenBank (2000). A link to the European Resource Center (RZPD) of the German Human Genome Project is given in an article by Lehrach (249).

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