Historical review

The Genesis of Microarrays

Michael C. Pirrung‡ and Edwin M. Southern§*

From the ‡Department of Chemistry, University of California, Riverside, CA, 92521

§Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU,

Abstract. This review provides a perspective on the initial development of microarray technologies by two independent groups in the late 1980s.

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*Address for correspondence to: ‡Department of Chemistry, University of California, Riverside, CA, 92521, USA. Fax: 951-827-2749. E-mail: michael.pirrung@ucr.edu. §E-mail: ed.southern@bioch.ox.ac.uk.

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Introduction

How does one become an inventor? Thomas Edison famously said that 'To invent, you need a good imagination and a pile of junk.' Well, sometimes. One of the authors (see below) favours this approach. But Edison's is not the only way nor is it the whole story. There are many different routes to invention and there is not just one kind of inventor. In this article we explore the inventive process through two examples which led to the same invention, the DNA chip or microarray, by different routes.

The essential element of invention is the concept or the idea which leads to something new. Most inventions arise by combining old ideas in new ways, so inventors tend to be people who are constantly seeking to understand the way that things work, storing a stock of concepts which can then be brought to creating new, useful combinations. Once the idea has been formulated, there follows a process of refinement, in which various routes to implementation are explored; and this can be conceptual, or experimental or, more usually, a combination of the two. The inventor will often seek to protect the invention by applying for a patent at some stage in this process. Finally, a successful invention will be put to use, and in most cases developed commercially.

It sometimes happens that an invention is used in different ways from those that motivated the inventor. The DNA microarray, originally developed for sequence analysis, became widely adopted when a different application was identified – the measurement of gene expression levels. In the intervening years, several other applications have become important – analysis of gene copy number, detection of chromosomal aneuploidy,

identification of protein binding sites, SNP and mutation detection, etc. [1]. In this article, we focus on the paths to the invention.

The late 1980s were a time when Maxam-Gilbert and Sanger sequencing were the most commonly used methods of single nucleotide analysis of nucleic acids. The grand challenge of the day was whole genome sequencing, but it was unclear what technology could be practical, in speed and cost, to achieve the ultimate goal, the sequence of the human genome. There were also many other nucleic acid analysis processes of the time that, certainly in retrospect, cried out for technological advancement. Microarrays were not necessarily developed to address any of these challenges, or even nucleic acids at all. DNA microarrays should be recognized as an early example of a technology that permitted a previously underutilized approach to biology to come to the fore - the systematic collection of large datasets to allow data mining to reveal intricate functions, rather than a hypothesis-driven search for an answer to a specific question. They also are the grandfathers of some of today's next-generation sequencing technologies [2].

This perspective will first describe separately how the two different groups approached this problem, and then aim to compare and contrast them in a concluding section. Other perspectives on how high-density DNA arrays came to be have been offered by historians [3] and discussed in more lay publications [4].

Oxford/Southern

A brief history of DNA hybridisation. In the 1960s, shortly after the description of the double helical structure of DNA, it was discovered that the two strands could be separated

by heating DNA in solution. Remarkably, duplex could be reformed; by lowering the temperature, the two strands came together to form perfect duplexes. This property was quickly exploited as a method for the detection and measurement of nucleic acids. In one popular method, spots of samples to be analysed were bound to a membrane support, which was then immersed in a solution of the probe, which had been labeled with a radioisotope: the presence and amount in each sample of sequences complementary to the probe were measured from the radioactivity picked up by the membrane [5].

The microarrays we describe here comprise a small glass 'chip' or microscope slide with a set of oligonucleotides printed on its surface. The oligonucleotides are printed in very small spots so that many thousands can be accommodated on one chip. We will describe methods of fabricating these arrays below. By contrast with the blotting method, it is the sample to be analysed that is in solution (usually labeled with fluorescent dyes) and applied to the array in a cassette under conditions that favour hybridisation between the target and the oligonucleotides. If there is correspondence between the sequence of an oligonucleotide in the array and a region of the target RNA or DNA in the sample, the spot will light up when viewed with illumination of the appropriate wavelength. This information can be quantitative, as is needed to measure how much of a mRNA is present in a sample; or it can be qualitative, +/-, which is enough to tell if a DNA sample contains a particular mutation.

The academic route to oligonucleotide microarrays. Alan Maxam and Walter Gilbert and Fred Sanger invented their famous sequencing methods in the mid to late 1970s. The methods transformed genetics and, arguably, put the whole of biological science on an entirely new footing. Sanger not only developed his sequencing method, but he also applied

it in a most interesting way. Rather than sequence 'interesting' regions of DNA, he selected small viruses and organelles – bacteriophage phiX174, the human mitochondrion, bacteriophage lambda – and sequenced them completely, thus providing a molecular framework on which to build an understanding of their biology. By the mid 1980s, molecular methods had been developed for mapping the human genome and it became clear that Sanger's strategy could be scaled up to sequence the human genome, with enormous potential for the understanding of human biology and disease. However, with a cost estimate of ~US$3Bn, the scale was beyond that to which biologists were accustomed at that time. It bore more resemblance to the ways of particle physicists, who were used to spending on this large scale. As it turned out, the first large scale sequencing of the human genome was carried out using modifications of Sanger's method. But in the mid 1980s, the community made a call for the development of new methods, particularly methods that could be applied to large genomes at a lower cost. To address this call, Akiyoshi Wada organised a meeting in Okayama of molecular biologists with an interest in technology development. At this meeting, one of his colleagues described a method for affinity purification of tRNA in which an oligonucleotide bound to a solid support in a column was used to capture the target molecule. They showed exquisite discrimination by eluting the column with a temperature gradient: the resolution was such that targets differing by a single nucleotide could be distinguished. It occurred to me that here was the basis of a potential sequencing method. If columns could be made with every possible sequence of a defined length, and used as described by Wada's group, then the sequence of a target could be reconstructed from the sequences of oligonucleotides it bound to, provided that the

lengths of the oligonucleotides were long enough that the chance of multiple occurrences in the target were small.[1] A few calculations showed that in order to sequence a meaningful length, one would have to use quite long oligonucleotides, and that would mean large numbers of columns – there are 4^N different sequences in a complete set of oligonucleotides of length N. While thinking about making support-bound oligonucleotides, I explored the chemistry that had been developed for oligonucleotide synthesis.

These explorations were very exciting. I discovered, first, that the methods for oligonucleotide synthesis were extremely efficient; it was possible to make quite long oligonucleotides in high yields by the repetition of two or three simple steps. Second, the favoured solid support for synthesis was porous glass beads. Uwe Maskos, a graduate chemist in my laboratory, quickly adapted this method of synthesis to make oligonucleotides which remained tethered to the glass after synthesis [6]. Uwe also showed that the oligonucleotides bound to glass beads could take part in hybridisation reactions. But how to make the large numbers of oligonucleotides needed for sequence analysis in a form that could do the hybridisations efficiently? Clearly, the column format was not well suited to this need. With blotting techniques in mind, it was natural to think of a flat substrate with dots of oligonucleotide probes attached, a format now known as the 'reverse

[1] Several other scientists had the idea of using oligonucleotide hybridisation around this time; the method became known as Sequencing by Hybridisation (SBH). Most of the proposals were based on the 'target down' approach. The exceptions were the gel pad method developed by Mirzabekov and colleagues, and the two methods described in this paper.

dot blot': since the chemistry was adapted to synthesis on glass, glass sheets became our preferred starting material. Uwe's molecule was used to initiate the synthesis; our first arrays were simple sets of oligonucleotides made on microscope slides.

To make arrays of different sequences, the chemicals must be directed to defined and separate regions on the glass surface. My colleague Martin Johnson adapted the pen of a pen plotter so that it delivered oligonucleotide precursors via a motor driven syringe pump, rather than ink from a reservoir, and John Elder programmed a computer to synchronise the movement of the pen with the action of the pump. Although this system worked, the spots were rather large and ill-defined because Uwe's chemistry created a surface that was readily wetted by the solvent. So we turned to a simpler, manual method, in which reagents were confined by barriers sealed against the surface. To make these devices, I drew on my 'pile of junk': in this case, silicone rubber tubing, which I could stick to the surface of a glass plate with silicone cement to create the wells and channels that we used to deliver reagents to the surface of the derivatised glass plate. With this system we quickly made simple arrays to test the basic method. The first array was simply different lengths of oligo-dT, to which we hybridised radiolabeled oligo-dA. More excitement: the hybridisation was rapid and efficient, and the background was clean. We then went on to develop arrays which represented the wild-type and sickle cell mutants of the beta-globin gene. Again, the results were clear. The signal over the mutant allele was much weaker than the wild type. At this point we knew we had a method with a wide range of potential applications such as the analysis of mutations in genetic diseases and SNP analysis on the large scale needed for mapping genomes and discovering disease-causing mutations. But what of the original,

more challenging aim of *de novo* sequencing? For this we needed to make arrays comprising all possible sequences of a given length. We developed a simple protocol for creating complete sets of sequences, using combinatorial principles, which could be realized by applying reagents to the surface of the support in rows and columns, flowing them in channels created from silicone rubber tubing. The protocol, which is illustrated in Fig. 1, is similar to the familiar way in which all the triplets of the genetic code are represented in a table. We used this protocol to make arrays of all 256 octapurines, combinations of As and Gs, in an eight step synthesis which we hybridised with radiolabelled, synthetic targets comprising known sequences of twenty Ts and Cs. My colleague, John Elder, devised an elegant method for reconstructing the sequence from the resulting autoradiographic image [7]. John's method compares the expected pattern of hybridization for every possible sequence with the actual hybridization results, which were quite noisey, and ranks them according to the closeness of fit. This novel and powerful method returned the correct result for the two sequences that were tested.

Other devices, using different geometries for the flow cells allowed us to make arrays of oligonucleotides of overlapping sequences that 'tile' through the sequence of a target. We used these arrays in several basic studies of the hybridization process, and to select effective antisense oligonucleotides for gene 'knockdown' experiments.

As observed above, there is more than one way to make arrays. In Oxford, we devised a method which used acid generated by an electrochemical reaction to remove the blocking group in the deprotection step of oligonucleotide synthesis in an approach related to that described by my coauthor below, which uses light to remove blocking groups. In the

electrochemical method, an array of electrodes printed on the surface of a silcon wafer is located close to the surface on which the oligonucleotides are synthesized, with a thin film of electrolyte between the two surfaces. At the deprotection step in the synthetic cycle, electrodes at positions where acid is required are switched to anodes for around 20 seconds. The reactions at the cathodes protect the regions under them from the action of the acid, so the features created by the anode reaction have sharp boundaries. The method is well suited to generating stripes of oligonucleotides, but the electrode geometry needed to make spots is more complex than we were able to create in our laboratory.

There came a point where the resources of our pile of junk and our workshop were overtaken by others with more advanced capabilities. Affymetrix developed their ingenious method, related below. Alan Blanchard in the laboratory of Leroy Hood built a machine which used inkjet heads to deliver reagents, much as we had used a pen plotter, but he succeeded in making features much smaller and better defined than we had achieved. His machine was taken up by Rosetta and is now the technology used by Agilent to make arrays on a large commercial scale.

Affy/Pirrung

Two companies were involved in the development of photolithographic DNA microarray technology. Affymax was founded in 1989, and Affymetrix spun off from it in 1993. Affymax was mainly interested in high-throughput drug discovery methods. A significant chemistry focus was peptides because they could be constructed in a building-by-blocks synthesis. This appealed to founder Alex Zaffaroni because it would enable large

collections to be prepared quickly to fulfill the needs of fast screening. One of the prominent library technologies of the day was the peptides-on-pins method of Geysen [8]. Its concept of identifying each sequence based on its position was appealing, but being based on the 96-well plate limited the number of peptides that could be prepared. It was our sense that comprehensive collections of peptides were needed, for example the complete set of pentapeptides (3.2 million sequences), and as powerful as Geysen's method was, it could not deliver that many peptides. I had only learned about the Geysen technology during a consulting assignment for Affymax in 1988, and was employed there during 1989.

In a January 1989 meeting with our peptides advisor, the late Murray Goodman, Affymax co-founder and vice-president J. Leighton Read, MD, reportedly inquired "Do you think we could make peptides the way they make computer chips?" That simple question spurred a flurry of activity. Read was aware that light was used to pattern semiconductors, made up of millions of miniaturized transistors, and envisioned a similar method to prepare millions of peptides. Goodman discussed with him the then-known use of light-sensitive protecting groups for peptide synthesis. I was splitting time between Affymax and my post at Stanford, so was unfortunately absent from this meeting, as I had a good deal of experience with synthetic photochemistry. However, Read and I met the next day to work through many of the concepts of peptide microarrays, and were sufficiently interested to prepare an invention record. A significant technological advance of that epoch in semiconductors was VLSI technology, an abbreviation for very large scale integrated circuits. Leighton Read dubbed our method VLSIPS, an acronym for very large scale immobilized polymer synthesis. The process we envisioned (Fig. 2) is conceptually distinct from Geysen's and

begins with a surface uniformly protected with photoremovable groups. Light would be directed to the sites on the chip at which a building block was to be added, removing protecting groups only there. The whole chip would then be exposed to a single building block that was protected with the same photoremovable group, recreating a uniformly protected surface. The process would be repeated at different sites with different building blocks, establishing different sequences at each site based on the patterning of light and sequence of reagents. Read envisioned that laser beams would travel to each tiny, targeted spot on the array to trigger *N*-protecting group removal. Wishing to test our concepts with a laser expert, we met with Richard Zare, my Stanford chemistry department colleague and one of the outstanding cadre of Affymax advisors. Zare's reaction was that lasers would not be a good choice, because laser speckle would blur the peptide synthesis spots, and because visiting each spot individually was inherently serial. He described the masking process used in semiconductor fabrication, where chemistry happens in many locations at once, in parallel. He also suggested glass as a readily available smooth surface and fluorescence or autoradiography as methods to detect the binding of target proteins to the peptide sequences generated at specific spots on the array.

As I began to work through the implications of our meeting with Zare, I realized that when using the masking approach, the number of masks needed to prepare a particular sequence (and, correspondingly, the number of coupling steps) would be one for each building block at each position in the sequence, whereas the number of sequences prepared would be the number of building blocks raised to the power of the length of the sequence (illustrated in Fig. 3). This characteristic, where the number of compounds made greatly

exceeds the number of steps, makes microarray fabrication a combinatorial synthesis [9]. Thus, it was conceivable that our 'holy grail' of the complete set of natural pentapeptides could be practically obtained, in only 100 steps (20 × 5). We could also calculate that the miniaturization afforded by the photolithographic approach (10 μm resolution was available at that time) could place this peptide library on an array of manageable size. A great deal of enthusiasm arose at Affymax for VLSIPS because of this potential, and it also seemed a particularly apt technology for a start-up biotech company located in Silicon Valley. This excitement was not universal, though, as some advisors opposed its development because they did not believe that quantitative yields would be possible in removal of a light-sensitive protecting group. As is well-known in solid-phase synthesis, it is essential to obtain as close as possible to 100% yields in both the coupling and deprotection steps to maximize the purity of the ultimate synthetic sequence. As it emerged, these concerns were also true for DNA arrays, and this problem has now been surmounted through the achievements of Beier and Hoheisel [10]. It also turned out that effective applications of photolithographic DNA arrays did not await the attainment of quantitative cycle yields, not the least because molecular biologists had yeoman experience getting useful data from nucleic acid probes that are not molecularly pure (to chemical standards).

Shortly after peptide arrays were conceived, our eyes were opened to the possibility of DNA arrays by a report of a workshop on human genome sequencing published in early 1989 [11]. Because genome sequencing was gaining a great deal of interest, methods that would be more capable of solving large genomes (much more powerful than Maxam-Gilbert sequencing, then state-of-the-art) were of high interest. In a side-bar to that report

was discussed a novel proposal, sequencing by hybridization, put forth by Yugoslav scientists [12]. It essentially involves reading DNA by words rather than by letters. It was envisioned to involve preparing filters bearing a plethora of sequencing targets and sequentially adding hybridization probes; the problem was the number of 8-mer probes required, of which there are 65,536. Sequencing by hybridization was criticized as "substituting one horrendous task {gel-based sequencing of thousands of cloned DNAs} for another,"[11] that is, preparing and hybridizing all of those probes. This problem excited both Leighton Read and myself because we saw VLSIPS as a way to readily provide the needed probes, which would require only 4×8 or 32 synthesis steps to make the 4^8 sequences. This task was far smaller than preparing the comprehensive set of pentapeptides, which I had already brashly stated could be accomplished within a year. With oligonucleotide probes already at known positions from the array fabrication, it was logical to propose simply conducting hybridizations to the array, one target at a time. This was similar to how we had envisioned binding experiments to peptide arrays, with one labeled recognition molecule competing among all surface sites bearing different peptide sequences. This way of using a DNA array would later be called 'format 2' sequencing by hybridization, and is how essentially all DNA microarray experiments are practiced today. We were not active nucleic acids researchers at this time or we might have recognized this as a 'reverse' blot; reverse, that is, of a typical DNA hybridization, where nucleic acid targets are distributed on a filter and a labeled probe is added to detect complementary sequences by position [5].

Another important connection for the VLSIPS team was made around this time, to Stanford electrical engineer Fabian Pease, an expert on photolithography who had no exposure to the world of biotech. After an initial meeting at which Read and I described the VLSIPS concept, he said something like "if what you told me about the peptide chemistry is true, this idea should work." His endorsement was a significant motivator for our efforts, as was his joining the Affymax board of advisors. His support became more manifest when his daughter-in-law, Ann Pease, began working at Affymetrix and made key contributions to the first published photolithographic DNA arrays (vide infra).

While employed at Affymax throughout 1989, I oversaw the further development of VLSIPS. Early in the year the company did not even have any laboratories, so we aimed to get initial proof-of-concept data by contracting with a local company, Metafluor. The first experiments performed there by Dennis Solas and Amy Liu involved an aminated polymeric membrane used in immunoassays. The amine was protected with a photoremovable analog of the carbobenzyloxy group. A shadow mask was created by drilling a metal sheet in the pattern of the Affymax logo, a stippled, stylized letter A. Exposing the membrane to UV light through the mask and then labeling it with an amine-reactive fluorescent dye gave a membrane that 'lit up' with the logo in green when exposed to a hand-held ultraviolet lamp. That membrane and lamp were used to illustrate the VLSIPS method as the Affymax team traveled internationally, seeking financing for the nascent company. The image was not microscopic, of course, since it had to be viewed with the naked eye. Our first patent application, filed in June, 1989, included this example; of course we were unaware that Southern had made his own filings on his method.

We recognized that the VLSIPS team needed physical scientists to address the issues of masked light delivery and the detection of binding on arrays, and turned to another of our advisers, UC-Berkeley's Richard Mathies, who had pioneered imaging technologies with another company, Molecular Dynamics. He recommended a postdoc in his lab, Steve Fodor, and we were (obviously) lucky to attract him to the company. He put together a state-of-the-art confocal imaging system and began making simple patterns. Even these early data showed that he could create molecular features at microscopic dimensions; the spatial resolution shown in Fig. 4 would enable all native octanucleotides to be placed on an array 13 mm square. Lubert Stryer had also by that time come on as the research director, and he and Steve made a great team on the biophysics side and in developing the mathematical formalism for designing masks, which are unlike any masks used in the semiconductor industry. The VLSIPS team experienced great success, including continued synthetic assistance from the Metafluor chemists after I relocated to Duke at the beginning of 1990. The team's results led to the first publication on VLSIPS in 1991 [13], which included mostly peptide arrays (including one of 1024 sequences) but also a patterned synthesis of a dinucleotide.

Many groups were tantalized by the prospect of DNA arrays following this disclosure, not the least being those developing the human genome project. A symposium on new sequencing technologies convened by the Department of Energy in Santa Fe later in 1991 was the first meeting between the authors of this review. It was also my first realization that microarrays had been independently conceived and developed, though the molecular

biology community had clearly been aware of Southern's work. His publications followed shortly thereafter.

Work at Affymax continued on peptide arrays, and Affymetrix was spun off to focus on the DNA array application. In early 1994, the first photolithographic DNA arrays were reported [14], with the first author being biophysicist Ann Pease. The technological advancements required for Affymetrix to deliver DNA microarrays to the marketplace were still substantial, however, stimulating others to develop alternative technologies to get DNA arrays into the hands of the biological community. The most prominent of these was spotted arrays that still have a significant presence [15], with their primary use being in gene expression profiling and other applications in RNA analysis.

What lessons can be drawn from my Affymax experience? That openness to cool new technologies and current scientific challenges stimulates the mind and makes unanticipated discoveries possible. That awareness of current technical advances outside one's field can lead to applications that were never envisioned in the original invention. That new technologies can be tested in simple ways that do not require elaborate supporting hardware (even when the ultimate application may require cutting-edge technology). That surrounding oneself with diverse scientists who are experts in their own disciplines can create invaluable synergies; to be trite, that the whole can truly be greater than the sum of the parts.

Epilogue

Why is invention regarded by many as a less noble pursuit than discovery, or the practical application of science less highly regarded than basic research? Perhaps it is because inventions are ephemeral, most have a few days in the sun before they are superseded: a good theory can be everlasting. But, then, most discoveries are made using inventions. Galileo needed his telescope to observe the satellites of Jupiter. Van Leeuvenhoek could not have made his discoveries without the aid of his microscope. Perhaps this attitude is deeply rooted in ancient societies' veneration of the prophet, the philosopher, the priest; while the common man received little recognition for his quiet labours in the field or the workshop. In modern times, we still need sages to guide us through the current geopolitical turbulence, of course. But we are faced with huge practical problems, too: the growing shortage of fresh water and of energy, and global warming among them. Who can deny the need for scientists and inventors to solve these huge problems? Unfortunately, the perception is sometimes spread that science and technology are the causes of our problems, rather their solution.

We can only hope that the coming generations will continue to produce scientists and inventors of the caliber that brought us the progress we enjoy today, a reason we believe perspectives such as this one are worthwhile to offer.

Fig 1. Schematic of a 6-, 7-, and 8-mer microarray made from two building blocks A and G by parallel synthesis using channels to limit reagents to only rows or columns. An array of sequences is illustrated that can be made in eight coupling steps when reagents are applied

to the surface in the order shown. In this layout, the upper left 8 × 8 square includes 6-mers, the 16 × 8 rectangles to its right and below it include 7-mers, and a 16 × 16 square includes 8-mers (as exemplified in the lower right of the figure). This process can be extended to any oligomer length by encompassing two rows/columns at each additional level. (from Maskos, U., Southern, E. M. (1993) *Nucleic Acids Res*. **21**, 4663-9 and Southern, E. M., Maskos, U. (1994) *J. Biotechnol*. **35**, 217-27, reproduced by permission of Elsevier and Oxford University Press)

Fig 2. Light-directed synthesis. A surface is required whose reactive groups N are protected with a light-sensitive group X. Light can be spatially directed to the surface using a variety of means to deprotect groups in specific locations/patterns. The whole surface can be exposed to coupling reagents (X-A, X-B) in the following step, but only sites that were addressed by light in the previous step will be coupled.

Fig 3. A sequence of masked light exposure and coupling steps leads to the production of all possible trimers of the two building blocks A and B. While the advantage of combinatorial synthesis here is small (6 steps make 8 sequences), the advantage increases with the number of steps. The relationship of steps to sequences is: n^l sequences in $n \times l$ steps, where n is the number of building blocks and l is the length of the oligomer. While originally conceived for peptide synthesis (n = 20), this combinatorial synthesis method is even more powerful for DNA, where only 40 steps could prepare 4^{10} oligonucleotides, or 1.05 million sequences. (this figure reproduced from the document archive of an author).

Fig 4. Confocal fluorescence microscopic image of an early prototype of a molecular array. This chip was made by UV exposure of a surface bearing nitrobenzyl carbamate-protected amines using a chrome-on-glass resolution test target as a mask, amine-reactive staining with fluorescein, and microscopy. The largest lines are ca. 50 μm wide.

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