DNA SEQUENCING

Massively Parallel Genomics

Stephen P. A. Fodor

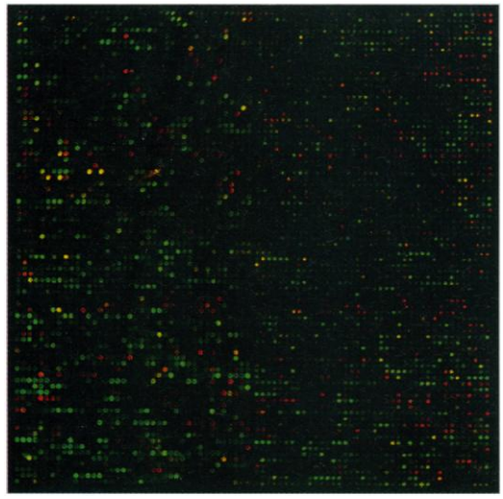
An enormous amount of genetic sequence data has been obtained with gel-based DNA sequencing methods. To understand the function of the genes and their health implications, genetic variation over vast numbers of cells, tissues, individuals, and organisms must be examined. The complex expression patterns of the estimated 100,000 genes comprising the human genome and the intricate developmental signals that define cellular fate will need to be understood. The magnitude of this analysis will dwarf the task of obtaining the primary sequence of the human genome, and so efficient means to experimentally access vast amounts of genetic information are critically needed.

TECH VIEW

Conventionally, researchers use analytical techniques to resolve sequence at the single-nucleotide level (*1*). In contrast, biological systems read, store, and modify genetic information, using the simple rules of molecular recognition. Each DNA strand carries the capacity to recognize uniquely complementary sequence through base pairing. The process of recognition, or hybridization, is highly parallel, and every sequence in a complex mixture can, in principle, be interrogated at the same time. Application of this highly desirable concept to sequence analysis has awaited new combinatorial technologies to generate high-density ordered arrays of large numbers of oligonucleotide probes (*2*).

At Affymetrix, we developed ways to synthesize and assay biological molecules in a highly dense parallel format. Integration of two key technologies forms the cornerstone of the method (*3*). The first technology, light-directed combinatorial chemistry, enables the synthesis of hundreds of thousands of discrete compounds at high resolution in precise locations on a substrate. The second, laser confocal fluorescence scanning, permits measurement of molecular interactions on the array. This technology is now commercial, with complete systems exported to dozens of sites.

Light-directed chemical synthesis employs two mature technologies: photolithography and solid-phase synthesis. Synthetic linkers modified with photochemically removable protecting groups are attached to a glass substrate. Light is directed through a photolithographic mask to specific areas of the surface to produce localized photodeprotection. The first of a series of chemical building blocks—hydroxyl-protected deoxynucleosides, for example—is incubated with the surface, and chemical coupling occurs at those sites that have been illuminated in the preceding step. Next, light is directed to a different region of the substrate by a new mask, and the chemical cycle is repeated. Highly efficient strategies can be used to synthesize any arbitrary probe at any discrete, specified location on the array in a minimum number of chemical steps. For example, the complete set of 4^N polydeoxynucleotides of length N, or any subset of this set, can be synthesized in only $4 \times N$ chemical cycles. Thus, given a reference sequence, a DNA chip can be designed that consists of a highly dense array of complementary probes with no restriction on design parameters. The amount of nucleic acid information encoded on the chip in the form of different probes is limited only by the physical size of the array and the achievable lithographic resolution. Current bulk manufacturing methods allow for ~409,000 polydeoxynucleotides to be synthesized on 1.28-cm by 1.28-cm chips.



Arrayed for sequencing. DNA chip fabricated by photolithography.

Photolithography allows the construction of probe arrays with extremely high information content. Because the array is constructed on glass, it can be inverted and mounted in a temperature-controlled hybridization chamber. A target sequence is fluorescently tagged and then injected into the chamber, where the target hybridizes to its complementary sequences on the array. Laser excitation enters through the back of the array, focused at the interface of the array surface and the target solution. Fluorescence emission is collected by a lens and passes through a series of optical filters to a sensitive detector. By simply scanning the laser beam or translating the array, or a combination of both, a quantitative two-dimensional fluorescence image of hybridization intensity is rapidly obtained. Commercial instrumentation for controlling the hybridization and scanning of the arrays, and software for image and data analysis have been developed. This approach requires only minute consumption of chemical reagents and minute preparations of biological samples.

An array of oligonucleotides complementary to subsequences of a target sequence can be used to identify a target sequence, measure its amount or relative expression level, and detect differences between the target and a reference sequence. Many different arrays can be designed for these purposes, and the applications appear to be only limited by imagination. The system consists of chips, a hybridization station to control hybridization, and a reader and software to access the chip data. Specific chip products for expression analysis, HIV array resistance screening, and gene resequencing are already on the market. Two versions of commercial readers are available: a first-generation system from Molecular Dynamics as well as a recently released high-performance system from Hewlett-Packard. Chip production is now in a scalable format. We are now producing ~5,000 to 10,000 chips per month, and we expect a large increase in production in the near future.

To fully understand gene expression, gene function, and the subtleties of regulation, the quantitative levels of expressed genes under various conditions must be assayed. In addition, if quantitative "snapshots" of gene expression can be captured, the dynamics of cellular pathways can then be deciphered. Recently, Lockhart *et al.* (*4*), published methods for the quantitative parallel measurement of cellular messenger RNA for gene sequences encoded on the chip solely from primary sequence data. RNAs present at a frequency of 1:300,000 were unambiguously detected with a quantitative assay spanning three to four orders of magnitude in concentration. Currently, Lockhart and group have developed chips containing the complete open reading frames from the yeast genome, a series of "custom" chips with hundreds to thousands of full-length genes or

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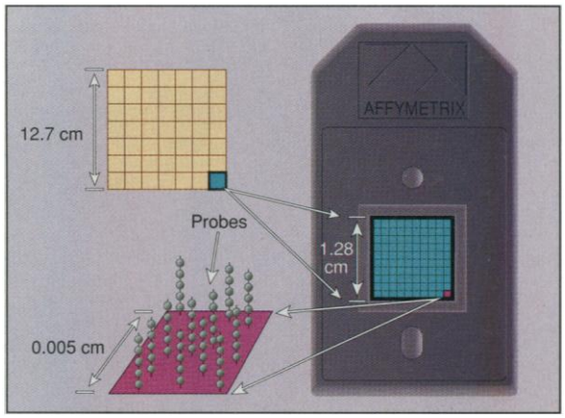
fragments from various databases, as well as "standard" chips containing more than 6,500 genes from the public databases. An expression chip with more than 50,000 expressed sequence tags from the public databases is currently in development.

This method complements other noncombinatorial array-based methods that involve the serial spotting of multiple clones or complementary DNAs (cDNAs) onto nylon membranes or modified microscope slides (5). These latter methods are inherently parallel for the analysis of sequence information, and they complement the probe-based arrays in their ability to use previously nonsequenced biological materials for the expression analysis. However, hybridization to large cDNA or PCR products can be thermodynamically more stable than to a series of shorter discriminating oligonucleotides and can yield confusing cross-hybridization signals when examining closely related genes, gene families, or other variants. Nonetheless, as experimentation continues, new ways around these problems will be found. In combination, the synthetic and mechanical techniques build a powerful new platform for parallel gene expression and offer large time and labor improvements over traditional cDNA library sequencing methods.



Making the matrix. Glass wafer is divided into DNA chips that contain the probe arrays.

Understanding the relationship between genotype and phenotype is a critical technical bottleneck in modern genetics. For example, consider examining 50 kilobases (kb) of coding sequence for 1000 individuals. The genes are known, but the prevalence, location, and identity of polymorphisms are not. The methods of conventional gel-based sequencing that are so effective in the initial gene sequencing are not efficient for this task. Comparative gel-based sequencing is indistinguishable from a de novo sequencing reaction, and so the de novo sequencing reaction must be carried out for all 50 kb over the 1000 individuals, or roughly 50 Mb of sequence. This illustrates the stark contrast of technical requirements when compared to a chip-based approach.

Chee *et al.* (6) recently showed how the entire human mitochondrial genome can be sequenced with high accuracy in a single hybridization experiment. A total of ~135,000 oligonucleotide probes were used to check the sequence of ~33 kb (forward and reverse strands) of the mitochondrial genome in one reaction. In addition, 179 of the 180 polymorphisms present in control samples were correctly detected. Two-color comparative sequence analysis experiments were performed that demonstrated how mutations or polymorphisms could be detected on a very large scale, making it now possible to use the technology for large-scale polymorphism screening efforts. At the current state of development, 1.28-cm by 1.28-cm chips can contain enough probes to scan anywhere from 32 kb to more than several hundred kilobases of sequence, depending on the specific chip design and accuracy requirements of the screen. Put in the context of the previously posed experiment, 1000 chips each containing 50 kb could easily and quickly perform the comparative sequence analysis.

Designing arrays to detect specific allelic variation is relatively straightforward. In addition to using chip designs appropriate to scan a sequence (as in the polymorphism application), blocks of probes can be dedicated to the specific detection of known allelic variation. Cronin *et al.* (7) designed a chip-based assay to detect multiple mutations in the CFTR gene, Kozal *et al.* (8) targeted HIV, Hacia *et al.* (9) examined the BRCA1 gene, and a number of new designs are in development for examination of p53 and cytochrome p450, and for microbial identification and antibiotic resistance. The amount of data coded on the array is limited only by the number of probes used per data point, the available synthesis area, and the synthesis resolution.

Recently, in collaboration with Lander's group at the Whitehead Institute (Cambridge, MA), Chee and Lipshutz have initiated work on a single nucleotide polymorphism (SNP) mapping chip (*10*). The immediate objective is to identify the common polymorphisms (those of ~20 to 50% frequency) contained within the mapped sequence-tagged site collection at the Whitehead Institute. These then form the basis set of biallelic markers that can be amplified from genomic DNA and applied to a probe array. Similar to the design used to detect allelic variation in the CFTR gene, blocks of probes are dedicated to each polymorphic form of the marker. This allows a straightforward detection of whether the sample is homozygous or heterozygous for each marker. These experiments offer enormous savings in time and labor, compared to standard gel-based microsatellite methods. Currently, prototype mapping chips containing ~500 markers are being produced, with plans to expand to a 2000-marker chip by the end of the year. These chips will be used for a number of applications, including studies of linkage, association, and loss of heterozygosity measurements.

The challenges of linking sequence variation to biological function are many. When the sequence is available, chips containing every gene in the human genome (chips for other genomes such as yeast have already been manufactured) can be produced, allowing genome-wide expression analysis. This should have a profound influence on the ability to elucidate the metabolic and disease pathways of the cell under a variety of developmental and environmental perturbations and have immediate applications in toxicology studies and pharmaceutical development. Screening chips will allow the databasing of large numbers of polymorphisms, and SNP chips will uncover how they are associated with disease. Chips providing insight into the genetics of model organisms have already been developed with strategies that will no doubt expand to include any organism of interest (*11*). DNA chip technology moves genetic sequence analysis away from serial gel-based methods to a massively parallel screening format. In time, technology will be needed to make this same paradigm shift for the hundreds of thousands of proteins, chemical messengers, and other molecular components of life.

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12. Supported in part by NIH grant P01HG01323.

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Jurassic Mouse

SIGHTINGS

In the movie *Jurassic Park*, an animated cartoon showed visitors of the fictional dinosaur zoo how ancient DNA could be injected into frog eggs to produce cloned baby *Tyrannosaurus rex* and velociraptors. That seemed far-fetched even for Hollywood, as the feasibility of just moving large pieces of intact DNA into an embryo has been highly questionable. Yet, recently, a group from Japan has for the first time succeeded in producing mice containing huge fragments of functioning human chromosomes (*1*). How did they do it?

There have been several experiments published over the past few years on the development of new technology to introduce ever larger pieces of human DNA into mice through in vitro manipulation. One driving force is the desire of scientists to introduce entire human genes into mice in order to study their phenotypes and other properties. The fact that most human genes are spread out over many tens to hundreds of kilobases of DNA presents a formidable technical challenge. The largest bacterial vector systems, for instance, can accommodate DNA of about 100 kb. Yeast artificial chromosomes (YAC) increase this by 10- to 20-fold. After substantial effort, scientists succeeded 3 years ago in producing a mouse containing YAC DNA (*2*). Tomizuka *et al.* (*1*) took an approach that bypassed all vectorology and just used fragments of human chromosomes directly. They designed experiments to find out if fragments of human chromosome 14, 22, or 2 could be transferred into and propagated in mice. They chose these chromosomes because they harbor the genes necessary to produce functional heavy λ, or κ chains of human immunoglobulin, respectively. A mouse with these loci could, in principle, make completely human-derived antibodies that would be useful for diagnostics and therapeutics.

Tomizuka *et al.* started by first randomly introducing a neomycin resistance gene by transfection into a population of human fibroblasts in culture. The Neo^r gene served to mark individual normal human chromosomes. Next, they fused these human Neo^r cells to a mouse cell line in culture. The human–mouse hybrids were then used to prepare microcells, or fragments of cells, containing pieces of individual chromosomes. The microcells were fused again to the same parent mouse cell line. This iterated process served to create individual mouse cell clones containing a human chromosome fragment or fragments. The use of microcells also meant that the large DNA was never really isolated outside any membrane, just shuttled between intracellular hosts. Because large DNA is difficult to prepare without shearing, this manipulation step was probably the key to success in the end.

To find those specific cell lines with chromosome 14, 22, or 2 DNA fragments, the authors used polymerase chain reaction amplification on individual clones with primers from various marker loci. Cells from positive clones were then fragmented into microcells and fused to mouse embryonic stem (ES) cells, cells which are pluripotent and can develop into any cell of a mouse. The resulting ES–Neo^r cells were injected into mouse embryos to produce chimeras. These chimeras were examined and bred to look for phenotypes and germ-line transmission of the human chromosome fragments.

To summarize, Tomizuka *et al.* found that the DNA from all three human chromosomes could be introduced into mouse chimeras. Using fluorescence in situ hybridization techniques, they could show that the human chromosomes propagated without any fusion to the mouse genome. By looking at the expression patterns in different organs, they showed that the immunoglobulin μ, κ, and λ genes were expressed mostly in spleen and thymus, proving that tissue-specific expression of these human genes is preserved in the mouse. Moreover, the genes underwent normal DNA rearrangements to form functional immunoglobulin subunits with junctions similar to those found in human cells. In fact, the mice could mount an immune response to a mouse antigen (human albumin) that included production of significant human immunoglobulin. Maybe even more striking was the fact that a fragment of human chromosome 2 (but not 14 or 22) could be passed intact through the germ line of several founder mice. Put another way, human chromosome 2 could successfully navigate through all stages of mouse mitosis and meiosis.

This work paves the way for more large, human DNA experiments in mice. For instance, the T cell receptor is an obvious candidate for reconstruction in this "simple" mammalian system. It is intriguing to think that complex human phenomena like cell-mediated immune responses to tumors and microbes can be dissected with these new tools. Also, in vivo recombination could be employed to create hybrid mouse–human chromosomes. Although the recent work will not lead to a bumper crop of neo-dinosaurs, it will probably lead to better model systems for biomedicine and biotechnology.

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–Robert Sikorski and Richard Peters

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Hitchhiking with Peptides

SIGHTINGS

One of the many challenges of gene therapy is the efficient delivery of DNA into specific target cells. At the moment, the most commonly used methods use viral vectors (usually retroviruses or adenoviruses), because they offer efficient delivery and can be targeted somewhat to specific cell types. Viral vectors, however, have several limitations: they often cause an intense immune reaction that rapidly clears them from the host, viral genomes can recombine during replication to form active disease-producing viruses, and viruses pose a possible cancer risk when they insert randomly in the genome. Scientists have thus been working hard to develop nonviral alternatives for intracellular DNA delivery.

Recent work by Niidome *et al.* (*1*) has now increased the armamentarium of the gene therapist. These authors synthesized amphiphilic peptides that can bind plasmid DNA and facilitate gene transfer. Their test system was the transfection of COS-7 cells with uptake measured by the expression of plasmid-linked luciferase activity. Amphiphilic peptides have both a hydrophilic and a hydrophobic surface. The size of the peptides they synthesized was between 12 and 24 residues. The researchers found that the transfection efficiency was higher with peptides that were longer or more hydrophobic, or both. It turns out that at the molecular level, these peptides form aggregates with plasmid DNA, as determined by circular dichroism and electron microscopy. These aggregates are, in turn, internalized by endocystosis. The fact that the transfection efficiency was increased by treating the cells with chloroquine, an inhibitor of lysosomal enzymes, supports this mechanism. The authors suggest that the most efficient peptides will be hydrophilic enough to electrostatically bind DNA, but hydrophobic

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enough to form peptide–DNA aggregates. One of the most interesting aspects of this work is that the ability of a peptide to form aggregates with DNA strongly predicts whether such peptide will be an efficient catalyst of transfection. The peptide's main role in the process is formation of the DNA aggregates rather than specific interaction with the cell membrane.

In the end, the best peptide they synthesized was as efficient for plasmid transfection in vitro as poly-L-lysine, but three and five times less efficient than calcium phosphate and cationic liposomes, respectively. Additional studies of the interaction of such peptides with DNA may offer further insight into the biochemical and biophysical factors involved in DNA transfection. In addition, because such peptides are easily synthesized and modified, it should be reasonably simple to design peptides with ligands recognized by specific cell surface receptors that could serve as targeting substrates in vivo.

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Robot Spotting

SITE FINDER

In addition to the techniques discussed by Fodor in the Tech View on page 393 on the use of photolithographic methods to produce DNA chips, other arraying strategies exist. One popular alternative involves the use of immobilized DNA fragments or clones as targets. DNA fragments (typically generated by polymerase chain reaction) can be spotted onto compact grids on glass slides, using custom tailored robotic systems. The resulting grids can be hybridized to fluorescent-labeled samples. Spots detected on the grid then represent genes whose DNA or RNA were contained in the samples. Analysis of signals obtained from messenger RNA probes can be used, for instance, to quantify gene expression.

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Saccharomyces cerevisae

One of the pioneers of this methodology is Patrick Brown at Stanford University (http://cmgm.stanford.edu/pbrown/). Researchers in his lab designed a Web site (http://cmgm.stanford.edu/pbrown/array.html) to demonstrate the technology and showcase

some recent results. Their Web site provides detailed protocols describing their microarraying technique, lists of the parts needed to build the apparatus, and even some short MPEG videos of the machine in action. They also present the results of a microarray that can detect all 6116 genes in the *Saccharomyces cerevisae* genome. The Web site is highly informative and distributes many of their tricks to the community at large.

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Browser Half-Life

NET TIPS

Would you use an isotope that has decayed by four half-lives to perform a critical experiment? Probably not. But most scientists today use Internet browser software that has decayed by as much relative to the state of the art. Given how we rely on the Net for database access alone, it would seem that even the brightest of us may be "browser deficient".

The technologies behind the Internet (the protocols and software languages) are evolving at an amazing speed. Just look at the original language upon which the World Wide Web was built, hypertext markup language (HTML). It is the HTML code that controls most of what you see when you browse the net, from the position of each word on the Web page to the color of the hypertext link. HTML specifications are set by the World Wide Web Consortium (W3C), a nonprofit organization. They are constantly being upgraded by W3C, with the current version of HTML at 3.2.

Now, add to HTML changes the fact that Web site developers are free to employ a variety of other software languages (Java, JavaScript, VBScript, VRML, and so on) to send their information, and you begin to see the challenges with Internet communications. Like HTML, these languages too are evolving, and newer versions require newer browser software or appropriate software plug-in modules. If the browser can't handle the Web site transmission, the appearance of the site can be odd or even blank.

As a specific example, version 1.1 of Netscape Navigator introduced an upgraded version of HTML that allows positioning of page elements in tables, much like a spreadsheet with invisible lines. As a result, Web sites that use table features will look strange when viewed with a Netscape browser that is not version 1.1 or later. Because most sites use HTML tables in some form these days, the scientist using an underpowered browser will be looking at the world through the equivalent of broken glasses.

Currently, there are only really two companies that dominate the browser market, Netscape (Navigator) and Microsoft (Internet Explorer). Browsers from these companies have the rich set of features that today's user will need. Like other software, browsers are released as numbered versions. Both companies have released fully debugged products labeled version 3 that can be downloaded on the Net. For most scientific sites on the Internet today, these 3.x versions from either company should be adequate for the next few months. (The "x" number increases as patches to existing bugs are made.) The latest 3.x products have been fine tuned through multiple bug-fix cycles and are more reliable. Given the speed with which software can be "shipped" via the Net, there is usually a lag time before any newly minted browser version is perfected. However, the pace of future development doesn't stop.

Both companies are already introducing a wealth of new features into their fourth-generation products, including nonstandard extensions to HTML that will extend the possibilities for those involved in Web page authoring. Adventuresome users who want to be at the cutting edge can dive into Netscape Navigator 4 (called Communicator) today, as it has just been fully released. In addition to support for the newest Net programming language versions, Communicator comes packaged with a host of additional modules for sending e-mail, reading newsgroups, and so forth. A version-4 preview release of Microsoft's browser is now available as well, with final versions ready later in the summer and fall. Different operating system versions may be shipped at different times. To learn more about the features of each new browser version, their availability, and where they can be acquired, point your existing browser to http://www.medsitenavigator.com/techsight/nettips_2.html where you can find detailed on-line documentation. We will revisit the browser issue in a later column to follow up on the state of this evolving technology.

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Miniature Temperature Data Logger

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PRODUCTS

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enzyme filters, summarized as text files, or listed together with DNA sequences. CloneMap can import sequences from formats such as GenBank, EMBL, GCG, and FastA, and can produce publication-quality graphics. It runs under Windows 3.1, Windows 95, and Windows NT. **CGC Scientific. For information call 314-207-0131 or circle 145 on the reader service card.**

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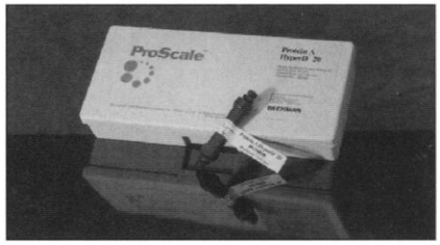

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Literature

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