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Maskless fabrication of light-directed oligonucleotide microarrays using a digital micromirror array

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Oligonucleotide microarrays, also called "DNA chips," are currently made by a light-directed chemistry that requires a large number of photolithographic masks for each chip. Here we describe a maskless array synthesizer (MAS) that replaces the chrome masks with virtual masks generated on a computer, which are relayed to a digital micromirror array. A 1:1 reflective imaging system forms an ultraviolet image of the virtual mask on the active surface of the glass substrate, which is mounted in a flow cell reaction chamber connected to a DNA synthesizer. Programmed chemical coupling cycles follow light exposure, and these steps are repeated with different virtual masks to grow desired oligonucleotides in a selected pattern. This instrument has been used to synthesize oligonucleotide microarrays containing more than 76,000 features measuring 16 μm². The oligonucleotides were synthesized at high repetitive yield and, after hybridization, could readily discriminate single-base pair mismatches. The MAS is adaptable to the fabrication of DNA chips containing probes for thousands of genes, as well as any other solid-phase combinatorial chemistry to be performed in high-density microarrays.

Keywords: oligonucleotide microarrays, digital micromirror device, DNA chips, maskless lithography, I-line, ultraviolet, optical lithography

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The current method of oligonucleotide microarray fabrication uses ultraviolet (UV) light passed through chrome/glass masks to direct the synthesis of oligonucleotides composed of photolabile phosphoramidite deoxynucleosides[1,2]. Photolithographic masks are used to control the light-directed oligonucleotide synthesis. Each chip consisting of N-mer oligonucleotides requires, in principle, $4 \times N$ masks. For example, a DNA chip of 25-mers may require 100 different masks, leading to high cost and long fabrication time. A maskless approach would circumvent the need for chrome/glass photolithographic masks, thereby reducing the cost and turnaround time for making custom DNA chips and greatly increasing their versatility.

We report a maskless technique for the light-directed synthesis of high-resolution oligonucleotide microarrays that uses a digital micromirror array to form virtual masks. The digital micromirror array used is Texas Instrument's (Dallas, TX) Digital Micromirror Device (DMD)[3], which consists of a 600×800 array of 16 μm wide micromirrors and is commonly used in computer display projection systems. These mirrors are individually addressable and can be used to create any given pattern or image in a broad range of wavelengths. With 1:1 imaging, this DMD can be exploited to address 480,000 pixels in a 10×14 mm area, theoretically allowing the synthesis of nearly half a million different oligonucleotide sequences. In addition, a self-contained system not requiring clean room operation can also be easily implemented.

Results

A schematic of the maskless array synthesizer (MAS) is shown in Figure 1. Each virtual mask was generated on a computer that relayed the image to the DMD. The MAS formed a UV image of the virtual mask on the active surface of the glass substrate mounted in a flow cell reaction chamber connected to a DNA synthesizer. A Köhler illumination system[4] was used to collect UV light from a 1,000 W Hg arc lamp (Oriel Instruments, Stratford, CT). A dichroic mirror and liquid filter excluded infrared radiation, which could heat and damage the optics or the DMD. In addition, a long-pass filter excluded wavelengths <340 nm that could damage the oligonucleotides[5]. A 0.08 numerical aperture reflective imaging system based on a variation of the 1:1 Offner relay[6] was used to form the image of the virtual mask.

Because the resolution requirements (16 μm squares separated by 1 μm borders) were not exceptional, it was possible to obtain acceptable optical performance for the imaging system at different points in the field defined by the DMD size. Therefore, in MAS, the reflective system was used in full-field imaging mode rather than in the scanning mode used in semiconductor photolithography. The performance of an optical system can be quantified using the modulation transfer function (MTF), an indicator of how well the system images a line pattern of increasing resolution[7]. The MTF of the MAS optical system was calculated using the optical system simulation package Code V (Optical Research Associates, Pasadena, CA and is shown in Figure 2A for field positions at the center and opposite corners of the 12×16 mm object modeled. All of the curves were well above the diffraction limit. A two-dimensional simulation of image formation using a 16 μm checkerboard as the object is shown in Figure 2B. To test the predicted performance, MAS was used to print 16 μm patterns in Shipley 1,827 resist, a common I-line (365 nm)

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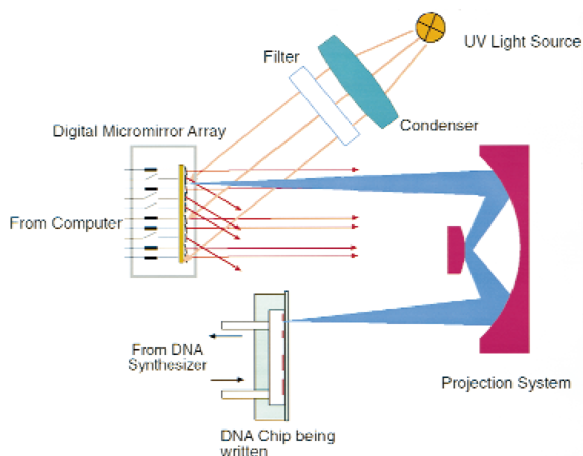

Figure 1. Schematic of the maskless array synthesizer (MAS). A Kohler illumination system is used to illuminate the digital micromirror array with UV light. A reflective Offner relay 1:1 imaging system with a numerical aperture of 0.08 forms an image of the pattern on the digital micromirror array on the active surface of the glass substrate. The glass substrate is enclosed in a flow cell connected to a DNA synthesizer.

photoresist used in the semiconductor industry. The transmission optical micrograph (Fig. 2C) shows that the checkerboard pattern was clear, with the lighter areas corresponding to the UV exposed regions. The sloped resist sidewalls formed during development scattered the illuminating light and therefore appear as dark borders around the mirror features. Figure 2C closely resembles the simulation in Figure 2B.

Patterning of photolabile substrates. The MAS was used to pattern photosensitive groups covalently attached to microscope slides. The photolabile protecting group (R,S)-1-(3,4-(methylenedioxy)-6-nitrophenyl)ethyl chloroformate (MeNPOC) was synthesized and attached to the nucleotides and hexaethyleneglycol (HEG), a spacer molecule, following a protocol reported by McGall and colleagues[8]. The photoprotected HEG was converted to a phosphoramidite following the aforementioned protocol, which was then covalently attached to a silanized microscope slide. This produced a microscope slide covered with a monolayer of spacer molecules containing hydroxyl groups protected by the photolabile MeNPOC group. Exposure to UV light resulted in the removal of the MeNPOC group, revealing free hydroxyl groups. A high-resolution pattern of UV light created with MAS and projected onto the microscope slide produced an identical pattern of free hydroxyl groups on the microscope slide. To visualize the pattern of deprotected hydroxyl groups, fluorescein amidite was added to the slide, resulting in coupling of a fluorophore to the free hydroxyl groups. The slides were then deprotected and imaged using a confocal microscope.

An exposure under an argon atmosphere (19.5 mW/cm^2, 4 min) was carried out, yielding the confocal micrographs shown in Figure 3A and B. A resolution test pattern consisting of 16 µm wide horizontal and vertical bars printed in MeNPOC-HEG is shown in Figure 3A. As can be seen, both horizontal and vertical features were sharp, indicating acceptably low astigmatism. Figure 3B and 3C show 16 µm^2 checkerboard features patterned under argon and dioxine respectively. The checkerboard was resolved, and in places it is also possible to observe the physical details of the micromirrors themselves, namely a spot in the center of each mirror representing the point of attachment for a post anchored to the underlying structure of the DMD.

Stepwise yield of the MeNPOC bases. An array that contained 76,800 features, each measuring 16 µm^2 and organized into 192 blocks of 400, was synthesized with MAS. Each block, designed to

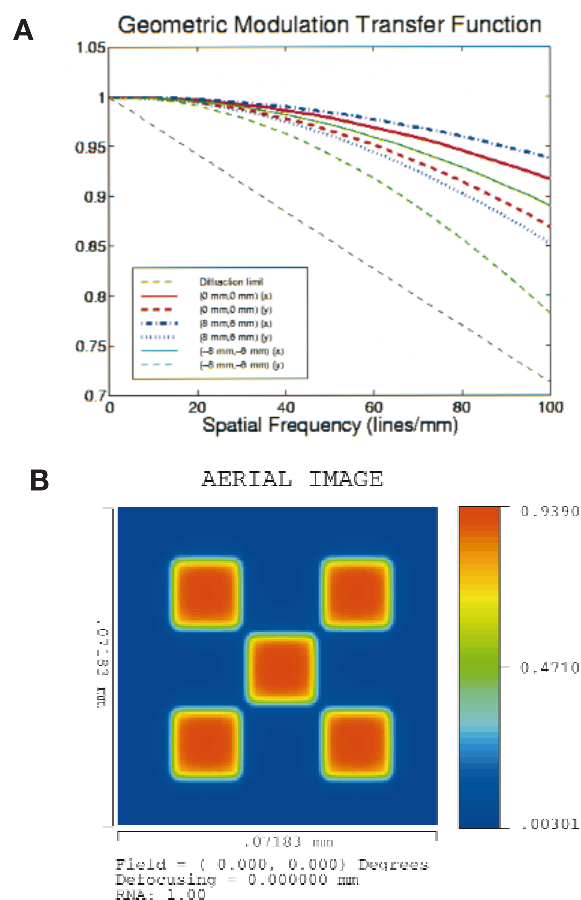

Figure 2. Optical performance of the MAS optical system. (A) Code V MTF showing high-contrast nearly diffraction-limited imaging for all field positions. (B) Code V two-dimensional simulation of image formation using a 16 µm checkerboard pattern as the object. (C) Transmission optical micrograph showing a 16 µm checkerboard pattern printed in Shipley 1,827 photoresist.

fill the field of view of the confocal microscope at 100× magnification, contained a border and identification number for orientation. Three different block designs were used: two for hybridization specificity data, and one for repetitive yield data. These blocks were repeated across the array to test the reproducibility of data in different regions of the array. In all, 82 different sequences were synthesized on the array.

After the different oligonucleotides were synthesized, all repetitive yield features, borders, and identification patterns received a final exposure to remove the last MeNPOC group. The array was then stained with fluorescein amidite. A confocal micrograph of one of the repetitive yield blocks is shown in Figure 4. A stepwise yield of 95% was calculated for the synthesis of the 18-mers using the Microarray Suite software from Scanalytics (Fairfax, VA). Blocks from all corners and the center of the array were analyzed, and there were no significant differences in repetitive yields.

In the hybridization blocks, four different 18-mers were synthesized and were designated as sequences 0, 1, 2, and 3. Sequence 0 was from the *Arabidopsis thaliana* Calmodulin-like domain Protein Kinase 6 (*CPK6*) gene, and sequence 3 was from the *CPK5* gene.

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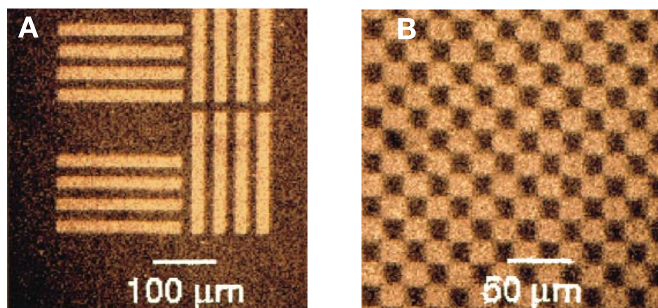


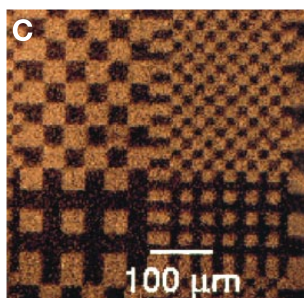


Figure 3. High-resolution patterning of MeNPOC using MAS. Resolution test patterns were printed on MeNPOC-HEG coated slides and stained with FluorePrime fluorescein amidite. (A) Confocal fluorescence micrograph of a resolution test pattern consisting of 1 micromirror pixel-wide (16 µm) horizontal and vertical lines. Both types of lines are sharp, indicating acceptable astigmatic correction. (B) Confocal fluorescence micrograph with each feature produced by a single 16 µm^2 mirror. (C) Confocal micrograph of pattern produced by exposing the MeNPOC-HEG in dioxane.

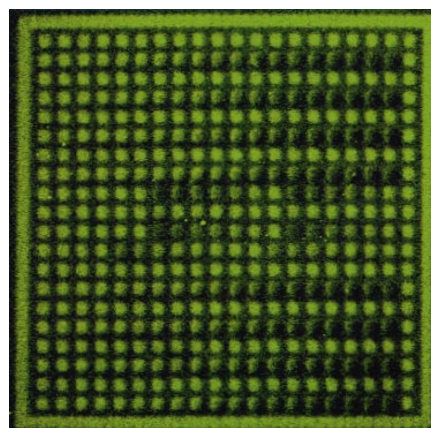


Figure 4. Repetitive yield of MeNPOC-bases. A micrograph of one of 192 blocks synthesized on a microarray. Four hundred 16 µm^2 features containing 82 unique sequences were synthesized to determine the repetitive yield of the MeNPOC bases. Different length oligonucleotides were synthesized and stained with fluorescein amidite. Relative stepwise yield was calculated from the fluorescence intensities of the different features.

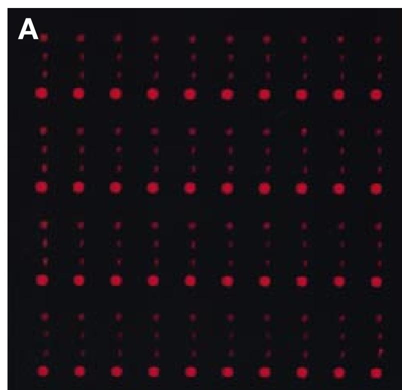
The *CPK6* and *CPK5* sequences varied from one another by three base changes; thus, we used the label 3 for the *CPK5* sequence, which signifies three mismatches from the 0 sequence (*CPK6*). The remaining two sequences varied from the 0 sequence by one and two bases. This design allowed us to test the ability of the array to discriminate between zero, one, two, and three mismatches.

After the repetitive yield data were obtained, the array was hybridized overnight with biotin-labeled target complementary to the *CPK6* sequence and stained with streptavidin phycoerythrin. The results of the hybridization are presented in Figure 5. Figure 5A shows a region of the array that contained the four 18-mer sequences repeated in a regular order. The bright spots contained the 0 sequence, and the three faint spots below each bright spot contained the 1, 2, and 3 sequences.

The hybridization characteristics of different length oligonucleotides are of interest for array design. Hybridization to the repetitive yield blocks provided information about specificity and sensitivity of different length oligonucleotides. Figure 6A is a confocal micrograph from hybridization of target complementary to the 0 sequence. The sequence for all four of the 18-mers was the same for the first five bases. The first mismatch occurred at the sixth base position in which the C in the 0 sequence was changed to a T in all the other sequences. The intensities of the four- and five-base oligonucleotides are equivalent, but the intensities of the six-base oligonucleotides are significantly different, with the 0 sequence being the brightest.

Discussion

Two of the major drawbacks of the Pease et al.[2] technique for fabricating oligonucleotide microarrays with photolithographic techniques are that the masks are expensive and require a significant amount of time to synthesize. The ability of MAS to create virtual masks that replace standard photolithographic masks overcomes both of these drawbacks. Using MAS, relatively inexpensive microarrays can be synthesized in about 12 h. Given sufficient



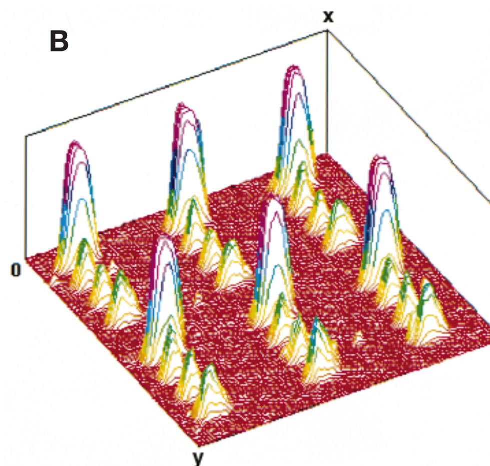


Figure 5. Hybridization specificity. (A) A micrograph of a hybridization of a biotin-labeled target (5′-biotinCTCTCGCTGTAATGACCT-3′) to a region of a microarray containing 160 16 µm^2 features with four different 18-mers. Rows 1, 5, 9, and 13 contain *Arabidopsis thaliana CPK6* sequence complementary (5′-AGGTCATTACAGCGAGAG-3′) to the target. Rows 2, 6, 10, and 14 contain the sequence (5′-AGGTCATTACAGTGAGAG-3′), which has one base change from the *CPK6* sequence. Rows 3, 7, 11, and 15 contain the sequence 5′-AGGTCATTATAGTGAGAG-3′, which contains two base changes from the *CPK6* sequence. Rows 4, 8, 12, and 16 contain the sequence 5′-AGGACATTATAGTGAGAG-3′, which contains three base changes from the *CPK6* sequence. After hybridization, the array was stained with streptavidin phycoerythrin. (B) A three-dimensional representation of a region of Figure 5A.

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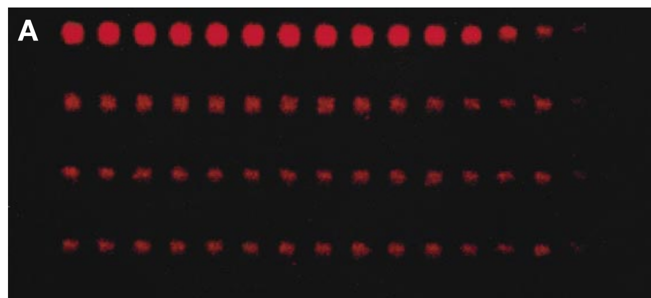

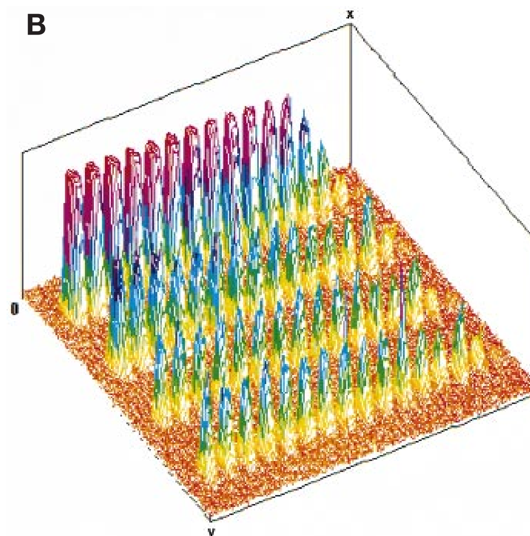

Figure 6. Hybridization of different length oligonucleotides. (A) A micrograph of a different region of the same array described in Figure 5. Rows 1 and 5 contain oligonucleotides with *CPK6* sequence ranging in length from 18 bases down to one. The 18-mer is the feature farthest to the left, and the oligonucleotides become one base shorter proceeding from left to right. Rows 2 and 6 contain one mismatch relative to the *CPK6* sequence. Rows 3 and 7 contain two mismatches relative to the *CPK6* sequence, and rows 4 and 8 contain three mismatches relative to the *CPK6* sequence. (B) A three-dimensional representation of a section of Figure 6A.

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computing resources to design the virtual masks, a new array based on new sequence from a sequencing project can be synthesized in <24 h. The cost of these arrays is also relatively low because there are no masks to produce and the primary expense is the chemistry. Currently, the MAS used in this report requires about $60 in chemicals for the synthesis of an array.

The resolution of commercial scanners is too low to adequately resolve the 16 μm^2 features MAS is capable of synthesizing. This necessitated the use of a scanning laser confocal microscope for data collection, which required that a significant portion of the array area be used for orientation features, thus reducing the number of experimental features. Improvements in scanner resolution and optimization of the optical resolution of MAS will allow all 480,000 features to be used for data acquisition. Construction of an MAS that contained Texas Instruments' new high-definition television mirror array[9] would allow synthesis of over 2 million features. Oligonucleotide microarrays commonly have 40 features for each gene; thus, probes for 50,000 genes could be synthesized on one array.

The size of the oligonucleotide features on the DNA chip can be adjusted by changing the MAS optics. For example, it is possible to use magnifying projection optics to print larger features. This would allow standard scanners (e.g., those used for spotted array-type DNA chips) to be used for data collection. Small feature sizes (0.35 μm) in photoresists are easily achievable using I-line photolithography reducing optics. Improving the sensitivity and resolution of the data collection are necessary to fully exploit the miniaturization possible with a future generation MAS.

Bioinformatics is an integral part of oligonucleotide microarray synthesis with MAS because of the computer-based design of the virtual masks. Software currently under development will allow for probe selection, layout, and design of the virtual masks, with the goal of allowing a user to submit a list of GenBank accession numbers or raw sequence, and to have a new array synthesized by the next day. Data analysis is complicated by the low resolution of commercial scanners. Currently, data are collected with a confocal microscope, and analysis is performed using the MicroArray Suite from Scanalytics, but the process is complicated by the need to stitch together the confocal images. However, it is likely that improved scanners that can adequately resolve 16 μm^2 features soon will be on the market.

Eventually, software could be developed that uses experimental results from one chip in the design of the next chip, such that a series of custom chips could be obtained, each of which depends on the results from its predecessor. This could benefit DNA sequencing projects, for example. The development of benchtop MAS could impact genomics research by making oligonucleotide microarrays readily accessible to scientists worldwide.

Experimental protocol

Silanization of microscope slides. Microscope slides were prepared according to the method of McGall and colleagues[8]. Briefly, soda lime glass microscope slides (Fisher Scientific Co., Itasca, IL) were soaked in a bath of Nanostrip (Cyantek, Fremont, CA) for 15 min, water, 1% (vol/vol) aqueous HCl for 1 min, 70°C 10% (wt/vol) aqueous NaOH for 3 min, water, 1% (vol/vol) solution of *N*-(3-(triethoxysilyl)-propyl)-4-hydroxybutyramide (Lancaster Synthesis, Windham, NH) for 15 min. The slides were then rinsed in 2-propanol for 5 min, water for 5 min, and then dried in a spin dryer for 5 min at 100°C under nitrogen. Slides were then stored desiccated at -20°C until use.

Synthesis of photolabile phosphoramidites. The MeNPOC-HEG-cyanoethylphosphoramidite (CEP) and MeNPOC-base-CEPs were synthesized according to the protocol described by McGall and colleagues[8]. Briefly, 3,4-(methylenedioxy)acetophenone (Aldrich Chemical Co., Milwaukee, WI) was reacted with glacial acetic acid and 70% (vol/vol) HNO_3 to produce methyl 3,4-(methylenedioxy)-6-nitrophenyl ketone. This molecule was reacted with sodium borohydride to produce (R,S)-1-(3,4-(methylenedioxy)-6-nitrophenyl)ethanol, which was then reacted with phosgene to produce (R,S)-1-(3,4-(methylenedioxy)-6-nitrophenyl)ethyl chloroformate (MeNPOC-Cl). This molecule was attached to hexaethylglycol and A(PAC), C(ibu), and G(ibu). These molecules were then converted to phosphoramidites by reacting with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite and diisopropyl ammonium tetrazolide to produce the final products.

Coating of silanized microscope slides with MeNPOC-HEG. Silanized microscope slides were attached to the reaction chamber and MeNPOC-HEG-CEP (50 mM) was delivered to the chamber via a standard DNA synthesizer (ABI 392), which was programmed to deliver reagents for DNA synthesis.

Patterned photodeprotection of MeNPOC. The MAS was used to deliver the desired photon using control software (Modularis, Chicago, IL) that designates the desired exposure time and displays the appropriate virtual mask at the correct time and place. The intensities given above for various experiments were measured at the image plane for an all-white image using an International Light UV Dosimeter (Newburyport, MA) with a calibrated detector for 365 nm radiation. A typical intensity was 19.5 mW/cm^2 for a lamp with 227 h of prior use. (Intensity increased to 28.5 mW/cm^2 with a new Hg arc lamp).

Staining of deprotected hydroxyl groups with FluorePrime fluorescein amidite. A mixture of FluorePrime (Amersham Pharmacia Biotech, Uppsala, Sweden) and DMT-Thymidine-CEP (Glen Research, Sterling, VA) 1:10 was diluted with anhydrous acetonitrile to a final concentration of 50 mM amidites. This mixture was delivered to the chamber via the DNA synthesizer during a cycle of standard phosphoramidite chemistry. After coupling of the Fluoreprime to the microscope slide, the slide was removed from the chamber. The slide was soaked for 15 min in a solution of ethylenediamine–EtOH

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(1:1) to remove protecting groups on the phosphate and fluorescein molecules and then rinsed for 5 min in water and spun dry.

Visualization of patterned fluorescein-labeled slides. The slides were viewed with a Bio-Rad (Bio-Rad Laboratories, Hercules, CA) MRC-1024 laser scanning confocal microscope. The 488 nm line of an Ar/Kr laser was the excitation source, and a 530 nm bandpass filter was used in front of a photomultiplier tube to collect the emission from the fluorescein. Images were acquired using both direct-mode and time-domain Kalman filtering of five image frames.

Oligonucleotide array fabrication and hybridization. The MAS was used to pattern MeNPOC-phosphoramidites in dioxane. The oligonucleotide 5′-AGGTCATTACAGCGAGAG-3′ was selected from the *Arabidopsis CPK6* gene using the Primer3 program[10] developed at Massachusetts Institute of Technology, Cambridge, MA). Three additional 18-mers were created by changing one, two, or three bases of the original sequence. After synthesis of the oligonucleotide probes, the slide was soaked in a solution of ethylenediamine–ethanol (1:1) for 2 h to remove the protecting group and then washed with water. The slide was hybridized with 200 μl of 100 nM synthetic biotin-labeled oligonucleotide target 5′-biotinCTCTCGCTGTAATGACCT-3′ (Genosys Biotechnologies, The Woodlands, TX). The hybridization solution contained 100 mM (2-[N-Morpholino]ethanesulphonic acid (MES), 1 M NaCl, 20 mM EDTA, and 0.01% (vol/vol) Tween 20. The hybridization was carried out overnight at room temperature in a hybridization chamber consisting of the DNA chip slide, a Kalrez (DuPont Dow Elastomers, Newark, DE) gasket, and a glass coverslip. After hybridization was complete, the array wash washed with a nonstringent wash buffer (6× SSPE [0.9M NaCl, 60 mM HaH_2PO_4, 6 mM EDTA (pH7.4)]) and 0.01% [vol/vol] Tween 20) and stained with streptavidin phycoerythrin (10 μg/ml) in 100 mM MES, 1 M Na^+, and 0.05% Tween 20 for 30 min. The array was then washed with the nonstringent buffer and imaged with a Bio-Rad confocal microscope.

Acknowledgments

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1. Fodor, S.P.A. et al. Light-directed, spatially addressable parallel chemical synthesis. *Science* **251**, 767–773 (1991).
2. Pease, A.C. et al. Light-generated oligonucleotide arrays for rapid DNA-sequence analysis. *Proc. Natl. Acad. Sci. USA* **91**, 5022–5026 (1994).
3. Sampsell, J.B. Digital micromirror device and its application to projection displays. *J. Vac. Sci. Technol.* **B12**, 3242–3246 (1994).
4. Welford, W.T. *Useful optics* (University of Chicago Press, Chicago; 1991).
5. Cadet, J. & Vigny, P. The photochemistry of nucleic acids. In *Bioorganic photochemistry: photochemistry and the nucleic acids* (ed. Morrison, H.) 1–237 (Wiley, New York; 1990).
6. Offner, A. New concepts in projection mask aligners. *Optical Engineering* **14**, 130–132 (1975).
7. Smith, W.J. *Modern optical engineering* 345–361 (McGraw-Hill, New York; 1990).
8. McGall, G.H. et al. The efficiency of light-directed synthesis of DNA arrays on glass substrates. *J. Am. Chem. Soc.* **119**, 5081–5090 (1997).
9. Gove, R.J. et al. High definition display system based on digital micromirror device. *Texas Instruments White Paper* (1997).
10. Rozen S. & Skaletsky, H.J. *Primer3.* (1998). Available at: http://www.-genome.wi.mit.edu/genome_software/other/primer3.html.

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