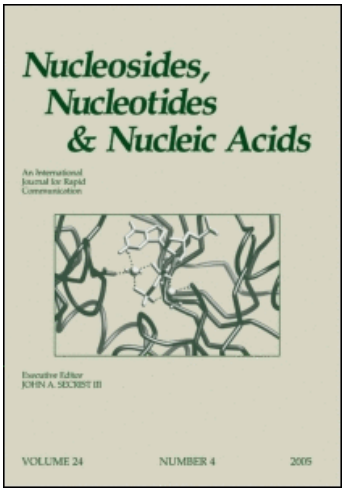

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PHOTOLITHOGRAPHIC SYNTHESIS OF HIGH-DENSITY OLIGONUCLEOTIDE PROBE ARRAYS

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ABSTRACT

High-density DNA probe arrays provide a massively parallel approach to nucleic acid sequence analysis that is transforming gene-based biomedical research and diagnostics. Light-directed combinatorial oligonucleotide synthesis has enabled the large-scale production of GeneChip® probe arrays which contain several hundred of thousand oligonucleotide sequences on glass "chips" about one cm^2 in size. Due to their very high information content, GeneChip® probe arrays are finding widespread use in the hybridization-based detection and analysis of mutations and polymorphisms ("genotyping"), and in a wide range of gene expression studies. The manufacturing process integrates solid-phase photochemical oligonucleotide synthesis with lithographic techniques adapted from the microelectronics industry. The present-generation methodology employs MeNPOC photo-activatable nucleoside monomers with proximity photolithography, and is currently capable of printing individual 10 μm^2 probe features at a density of 10^6 probes/cm^2.

High-density polynucleotide probe arrays have created a new paradigm for genetic sequence analysis, which is having a major impact on the future of biomedical research (1). These arrays are comprised of large sets nucleic acid probe sequences immobilized in defined, addressable locations on the surface of a substrate, and are capable of reading unprecedented amounts of genetic information from biological

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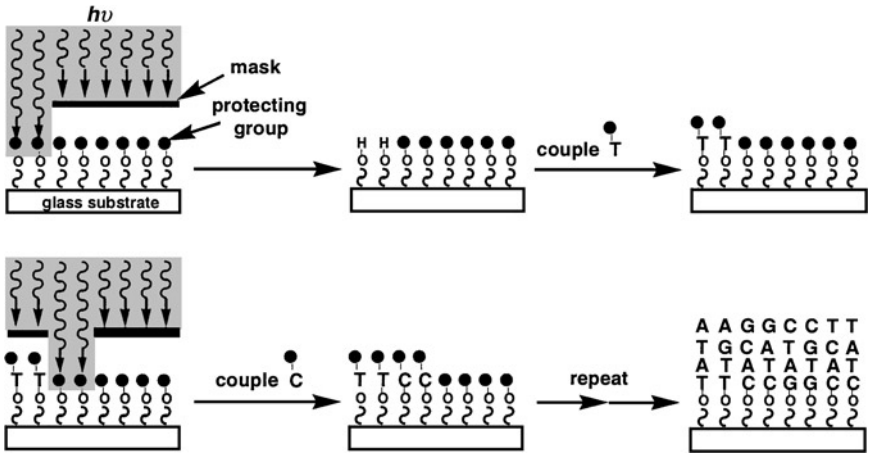

Figure 1. Photolithographic synthesis of oligonucleotide arrays.

samples in a single hybridization assay. The advent of this technology has relied on developing methods of fabricating arrays with sufficiently high information content and density. Light-directed synthesis (2–5) has enabled the large-scale manufacture of arrays containing hundreds of thousands of oligonucleotide probe sequences on glass "chips" about one cm^2 in size. In this process, 5′- or 3′-terminal protecting groups are selectively removed from growing oligonucleotide chains in pre-defined regions of a glass support by controlled exposure to light through photolithographic masks (Fig. 1). A planar glass substrate is first covalently modified with a silane reagent to provide hydroxyalkyl groups from which probe synthesis can be initiated (Fig. 2). These sites are extended with a polyethyleneoxide linker protected

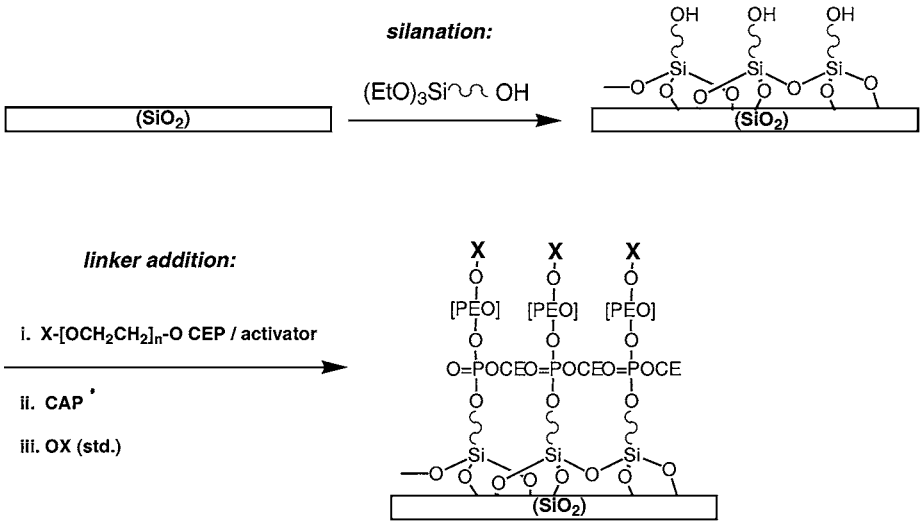

Figure 2. Preparation of glass substrates for light directed synthesis of oligonucleotide arrays.

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with a photolabile protecting group such that when specific regions of the surface are exposed to light, they are selectively "activated" for the addition of nucleoside phosphoramidite monomers. The monomers, which are also protected at the 5′ (or 3′) position with a photolabile group, are coupled to the substrate using standard phosphoramidite DNA synthesis protocols (4). Cycles of photo-deprotection and nucleotide addition are repeated to build the desired 2-dimensional array of sequences.

The photolithographic process allows parallel synthesis of large sets of probe sequences, and provides a very efficient route to high-density arrays. A complete set, or any subset, of all probe sequences of length "n" requires, at most, $4n$ synthesis steps. Mask sets can be designed to make arrays of oligonucleotide probe sequences for a wide variety of applications. Typical arrays are comprised of customized sets of probes that are 20–25 bases in length. Semiautomated manufacturing techniques, similar to those used in the microelectronics industry, have been adapted for the large-scale commercial production of GeneChip® array in a multi-chip wafer format.

The spatial resolution of the photolithographic process determines the maximum achievable density of the array and therefore the amount of sequence information that encoded on a chip of a given physical dimension. At present, GeneChip® arrays have individual probe features 20×20 microns in size on a 1.6 cm^2 chip, but this technology has proven capability for fabricating arrays with densities $>10^6$ sequences/cm^2, corresponding to feature sizes of less than 10×10 microns.

Current methodogy employs nucleoside monomers protected with a photoremovable 5′-(α-methyl-6-nitropiperonyloxycarbonyl), or "MeNPOC" group (Fig. 3) (3, 4), which offers a number of advantages for large scale manufacturing. MeNPOC phosphoramidite monomers are relatively inexpensive to prepare, and photolytic deprotection is induced by irradiation at near-UV wavelengths ($\phi \sim 0.05$; $\lambda_{max} \sim 350$ nm) so that photochemical modification of the oligonucleotides, which absorb energy below ~320 nm, can be avoided. The photolysis reaction involves an intramolecular redox reaction and does not require any special solvents, catalysts or coreactants. Complete deprotection requires less than one minute using filtered Hg I-line (365 ± 10 nm) emission from a commercial 1 kW

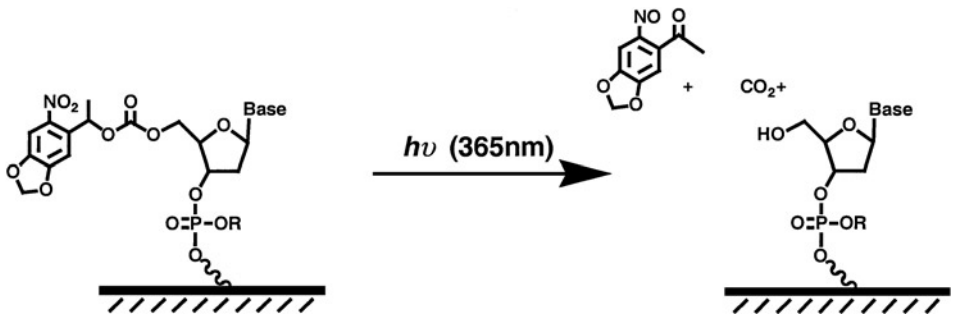

Figure 3. Spatially-addressable deprotection using the photolabile MeNPOC protecting group.

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photolithographic exposure system. The independence of photolysis rates on either the nucleotide base or oligomer length, conveniently allows the use of a single exposure setting for the entire process.

Photochemical deprotection rates and yields for oligonucleotide synthesis can both be monitored directly on planar supports using photolithographic procedures based on surface fluorescence (4). We have also developed a sensitive assay in which test sequences are synthesized on a support designed to allow the cleavage and quantitative analysis of labeled oligo products by IE-HPLC using fluorescence detection (Fig. 4). This method involves photolithographic synthesis of test sequences after the addition of a base-stable unsymmetrical disulfide linker and a fluorescein monomer to the support. The disulfide linker remains intact through synthesis and deprotection, but can be subsequently cleaved under reducing conditions to release synthesis products which are uniformly labelled with a 3′-fluorescein tag. This enables the direct quantitative analysis of synthesis efficiency using HPLC analysis with fluorescence detection. The sensitivity of the fluorescein label is necessary, since the amount of DNA synthesized on flat substrates (0.1–100 pmole/cm^2) is too low to detect by other means.

The average stepwise efficiency of light-directed oligonucleotide synthesis process is limited by the yield of the photochemical deprotection step which, in

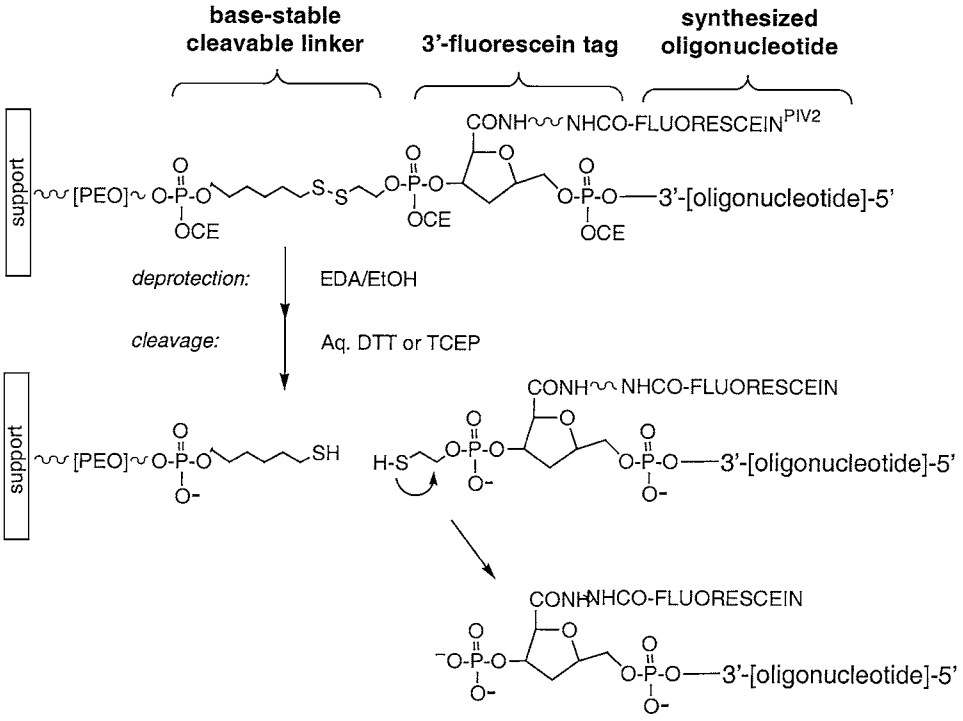

Figure 4. Method for fluorescent labeling and cleavage of DNA prepared photolithographically for subsequent characterization by HPLC.

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the case of MeNPOC nucleotides, is ~90% (4). The other chemical reactions involved in the base addition cycles (coupling, capping, oxidation) use reagents in a vast excess over surface synthesis sites, and given sufficient reagent concentrations and time for completion, they are essentially quantitative. Sub-quantitative photolysis yields lead to incomplete or "truncated" probes, with the desired full-length sequences representing, in the case of 20-mer probes, approximately 10% of the total. For several reasons, this has a relatively minor impact on the performance characteristics of arrays when they are used for hybridization-based sequence analysis. Firstly, the silanating agent described above provides abundance of surface synthesis sites (~120 pmole/cm^2), so the absolute amount of completed probes on the support remains high. Increasing the synthesis yield through alternate chemistries or processes available can increase the surface concentration of full-length probes. However, this can actually *decrease* hybridization signal intensity due to the steric/electrostatic repulsive effects that result when oligonucleotides are too closely spaced on the support. There is an optimum probe density for maximum hybridization signal. Secondly, array hybridizations are typically carried out under stringent conditions such that hybridization to significantly shorter ($<n-4$) oligomers is negligible, and statistically, full-length probes are the major species able to hybridize under such conditions. These factors, combined with the use of comparative intensity algorithms for data analysis (5), allows highly accurate sequence information to be "read" from these arrays with single-base resolution.

A number of alternate photolabile protecting groups have been described which may also be applicable to light-directed DNA array synthesis (6–9). We

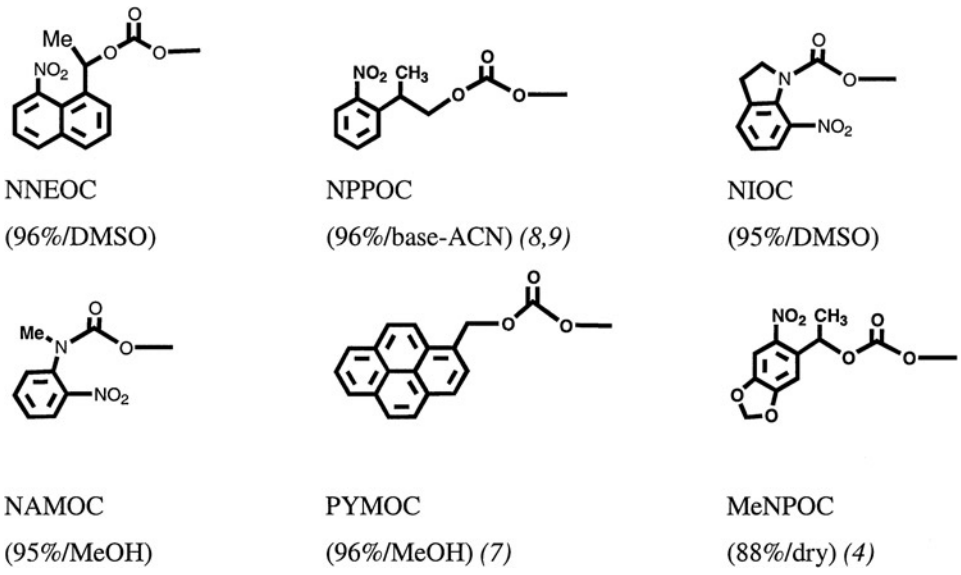

Figure 5. Photoremovable protecting groups for photolithographic DNA synthesis including optimum photolysis conditions and stepwise efficiency.

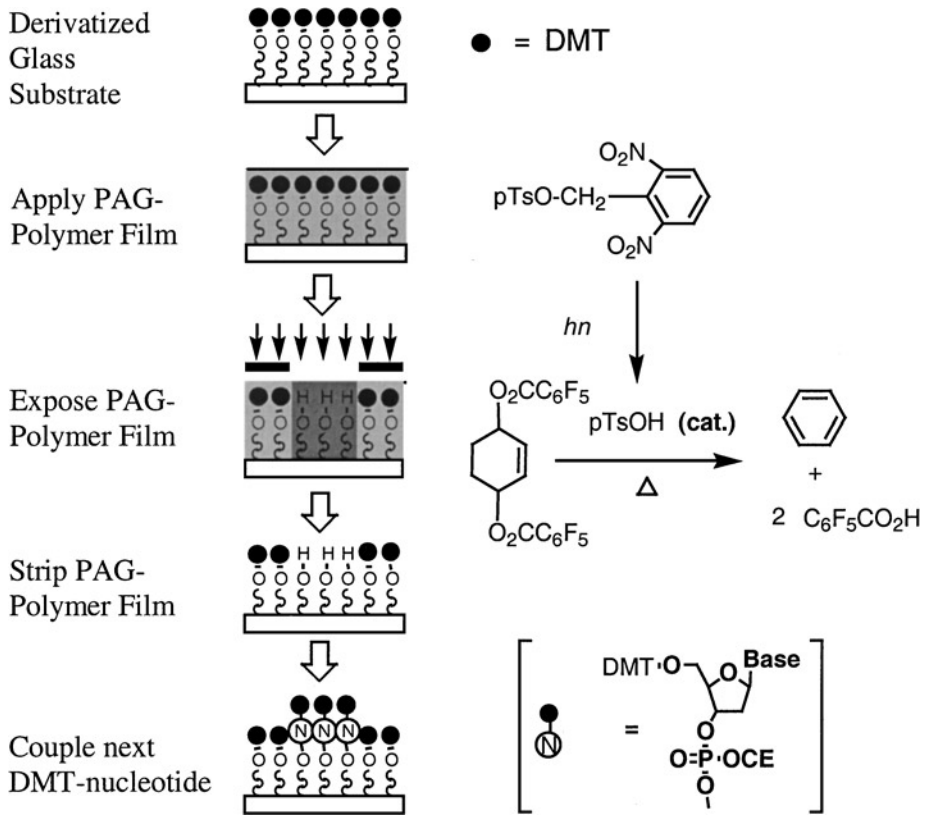

Figure 6. DNA probe array synthesis using photoacid generation in a polymer film to remove acid-labile 4,4′-dimethoxytrityl (DMT) protecting groups.

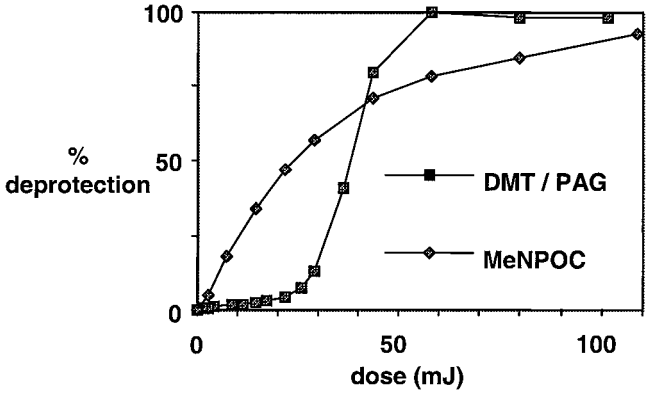

Figure 7. A comparison of photolysis dose-response curves of MeNPOC direct photoremoval, and DMT removal with a photo-generated acid in a polymer film.

have evaluated the synthesis efficiency of these compounds, as well as a number of previously unreported protecting groups, using the analysis method outlined in Figure 4. Some have the capability of providing higher stepwise coupling yields than the MeNPOC group (Fig. 5). However, achieving optimum yields with these groups typically requires immersion of the substrate under a layer of a solvent +/− catalyst during photolysis, and this is not compatible with high-throughput manufacturing processes based on high-resolution proximity or contact lithography.

In order to achieve higher photolysis rates, synthesis yields, and spatial resolution, we have developed photolithographic methods for fabricating DNA arrays which exploit polymeric photoresist films as the photoimageable component (10–12). These have the advantage of utilizing conventional DMT-protected nucleotide monomers. One such approach uses a polymer film containing a chemically amplified photo-acid generator (PAG), wherein exposure to light creates localized acid development adjacent to the substrate surface, resulting in direct removal of DMT protecting groups from the oligonucleotide chains (Fig. 6). This resist-based process provides stepwise synthesis yields of 98%, high-contrast with resolution capability below 10 microns, and photospeeds >10-fold faster than that achievable with standard photoremovable protecting groups (Fig. 7).

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