

Production by quantitative photolithographic synthesis of individually quality checked DNA microarrays

Markus Beier* and Jörg D. Hoheisel

Functional Genome Analysis, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 506, D-69120 Heidelberg, Germany

Received November 2, 1999; Revised and Accepted December 17, 1999

ABSTRACT

For DNA chip analyses, oligonucleotide quality has immense consequences for accuracy, sensitivity and dynamic range. The quality of chips produced by photolithographic *in situ* synthesis depends critically on the efficiency of photo-deprotection. By means of base-assisted enhancement of this process using 5′-[2-(2-nitrophenyl)-propyloxycarbonyl]-2′-deoxynucleoside phosphoramidites, synthesis yields improved by at least 12% per condensation compared to current chemistries. Thus, the eventual total yield of full-length oligonucleotide is increased more than 10-fold in the case of 20mers. Furthermore, the quality of every individual array position was checked quantitatively after synthesis. Subsequently, the quality tested chips were used in successive hybridisation experiments.

INTRODUCTION

Over the last few years, DNA microarrays have made their appearance in many fields of the life sciences, from biology and molecular medicine to quality assessment procedures (1). Their unique feature is the assembly of many DNA sensors on a small surface, resulting in the high degree of parallelism which is essential for large-scale genomic analyses. The specific configurations of current arrays are as wide as is the range of applications. However, in nearly all cases a set of either oligonucleotides or PCR products is attached to a flat solid support. While both formats can be generated by immobilisation of prefabricated molecules, the former can also be produced by direct *in situ* synthesis on the chip. An especially versatile procedure to this end is the technique of controlling synthesis by photolithographic methods derived from semiconductor technology. Photolithography combines the power of producing oligomer arrays of extremely high density and flexible patterns with a relatively simple procedure for independently directing the sequence of the molecules synthesised at the individual array positions. In addition, it facilitates large-scale chip production. Recent publications indicate the power of chips produced by this means in applications such as transcriptional profiling (2), analysis of single nucleotide polymorphisms (3) or disease-relevant mutations (4) and functional studies on deletion mutants (5), to mention a few.

Pioneered by scientists at Affymax (6,7), photolithographic DNA synthesis uses rapid solid phase phosphoramidite chemistry. Positional and sequential control are achieved by a combination of 5′-photoprotected phosphoramidites, which can be activated by irradiation with light, and a set of masks containing holes at appropriate positions. By placing a mask on the chip surface, only distinct areas are illuminated. Upon excitation, the photoprotecting groups of the already existing, partial oligonucleotide sequences at these locations, synthesised earlier during the process, are removed and the oligomers are extended by another nucleotide after adding the relevant monomer. Among the many factors responsible for the success of photolithographic synthesis, are three dominant ones: the accuracy in consecutive alignment of the masks, the efficiency of removal of the photoprotecting groups and the yields of the phosphoramidite coupling step. The first aspect is a matter of mechanical and optical instrumentation and relies on the appropriate adaptation of technology originating from semiconductor chip production. Chemically, the coupling yields and the efficiency of photoremoval of the 5′-photosensitive protecting groups are quality determining factors. To date, either [(α-methyl-2-nitropiperonyl)-oxy]carbonyl (MeNPOC; 8,9) or dimethoxybenzoincarbonate (DMBOC; 10) protecting groups (Fig. 1) have been employed. The former is currently used as the moiety of choice for the production of commercial DNA arrays made by Affymetrix. Although sufficient for the production of chips used in the above mentioned studies, the existing chemistry nevertheless limits performance, since stepwise yields of <90% are obtained (9), resulting in total yields of a few percent full-length oligonucleotides.

Recently, a new class of photosensitive 2-(2-nitrophenyl)-ethoxycarbonyl groups was developed for the protection of nucleoside 5′-hydroxyls (11), with the 2-(2-nitrophenyl)-propoxycarbonyl (NPPOC) group (Fig. 1) being a particularly promising candidate. Initial studies in aqueous methanolic solution showed that the NPPOC group could be very effectively removed from 5′-*O*-protected thymidine derivatives by irradiation at 365 nm (12).

Because of the non-quantitative yields of current *in situ* synthesis chemistries, with all the consequences on performance, and in recognition of the crucial role of the photoremoval process in this respect and, thus, the high potential of improving yields significantly by enhancing this step, a procedure was established based on the NPPOC chemistry that produced high quality oligonucleotide arrays by photolithographic *in situ* synthesis. In addition, processes were established to check

*To whom correspondence should be addressed. Tel: +49 6221 424684; Fax: +49 6221 424682; Email: m.beier@dkfz-heidelberg.de

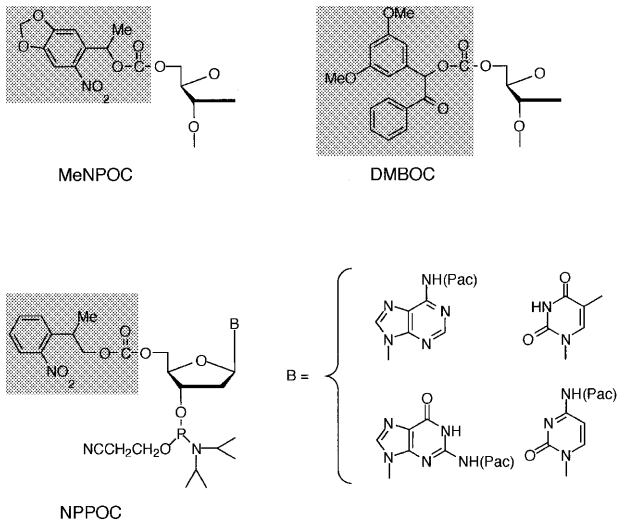

Figure 1. Photolabile protecting groups used in light-directed DNA array syntheses. Pac designates the phenoxyacetyl protecting group.

online the results of synthesis at each individual grid position prior to use of the chip in repeated hybridisation analyses.

MATERIALS AND METHODS

All chemicals and solvents were purchased from Fluka (Germany) or SDS (France), unless stated otherwise, and were used without additional purification. Photolithographic DNA array synthesis was performed on glass microscope slides (Menzel Gläser, Braunschweig, Germany) derivatised as reported in detail earlier (13). NPPOC-deoxynucleotide phosphoramidites were obtained from NIGU-Chemie (Waldkraiburg, Germany); MeNPOC-deoxynucleotide phosphoramidites were a kind gift from W. Pfleiderer (University of Konstanz).

Photolithographic DNA chip synthesis

Synthesis was carried out on a custom built synthesiser, consisting of a modified Eppendorf D 200 DNA synthesiser with opto-sensors to which a flow cell was connected. The flow cell was mounted on an optical bench for quasi-automatic, light-directed synthesis. Besides changes of the individual masks, all operations were automatic and PC controlled. Phosphoramidite coupling was done with 75 mM phosphoramidite solution in acetonitrile for 180 s. As the activator of choice, 0.5 M pyridine hydrochloride in acetonitrile was used. Oxidation was with 50 mM iodine in a solution made of acetonitrile, pyridine and water, mixed in a ratio of 7:1:2. For removal of the temporary 5′-photoprotecting groups, the flow cell was either flushed during light exposure with 50 mM piperidine, 50 mM diisopropyl-ethylamine or 50 mM 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU) in acetonitrile (NPPOC chemistry) or argon for removal under dry conditions (MeNPOC chemistry). Irradiation was from the reverse side using a 100 W Hg high pressure lamp (Leica, Bensheim, Germany) for a total of 5 min. After half the period, the flow cell was briefly washed and refilled with the appropriate medium for the second half of irradiation. By placing an interference filter (Owis, Stauffen, Germany) into the light path, only light of a wavelength of 365 nm was used. To compensate for any inhomogeneous irradiation across the cell, all syntheses were carried out repeatedly with the orientation of the patterns changed.

Determination of surface photolysis rates

All four premixed phosphoramidite monomers (dA, dC, dG and dT) were coupled to an aminoalkyl-silanated glass microscope slide. The resulting photoreactive surface made of NPPOC monomers was irradiated in the presence of 0.05 M DBU for increasing time intervals (10–900 s). Deprotection of MeNPOC monomers was done identically but in the absence of any liquid. Upon coupling with a mixture of 5 mM Cy5-phosphoramidite and 45 mM 5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxythymidine-3′-*O*-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite (MWG Biotech, Ebersberg, Germany), the deblocked 5′-termini were fluorescently labelled.

Quality control

For *permanent* labelling of the 5′-ends of newly synthesised oligonucleotides, 75 mM Cy5-phosphoramidite (Pharmacia Biotech, Freiburg, Germany) in acetonitrile was added as the final synthesis step, applying the same conditions for condensation and oxidation as with the photosensitive phosphoramidites. Subsequently, the DNA array was thoroughly washed with acetone/methanol/water (1:1:1) and 0.9 M NaCl, 60 mM NaH_2PO_4, 6 mM EDTA, pH 7.5, prior to fluorescence scanning analysis.

For a *removable* dansyl label, 75 mM dansylethyl-phosphoramidite in acetonitrile was added in a final step. A *removable* Cy5-label was produced by a two-step procedure of successive condensations with 75 mM 2-[2-(4,4′-dimethoxytrityloxy)-ethylsulfonyl]ethyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite (Eurogentec, Liege, Belgium) and 75 mM Cy5-amidite (Pharmacia Biotech). The Cy5 monomethoxytrityl group was removed by a 180 s reaction with 3% (w/v) trichloroacetic acid in dichloroethane. Subsequent to the fluorescence scanning analysis, the label was removed by a 2 h treatment with concentrated aqueous ammonia, which also cleaved the base- and phosphate-protecting groups.

Hybridisation

Hybridisations to the oligonucleotide arrays were carried out with 200 nM target DNA labelled with either Cy3 or Cy5 (ARK Scientific, Darmstadt, Germany) in SSARC buffer [600 mM NaCl, 60 mM Na citrate, 7.2% (v/v) Na-sarcosyl] at 15°C. Typically, 50 µl of the hybridisation solution were applied and spread evenly with a coverslip. Usually, hybridisation was overnight. Subsequently, the slide was carefully washed with cold SSARC buffer and ethanol to remove remaining salt residues and finally dried under a stream of nitrogen prior to signal detection.

Imaging and quantification

Fluorescence scanning was performed on a ScanArray 3000 system (General Scanning, USA). Quantification of signals was done with the Imagene software package (BioDiscovery, Canada).

For comparison of the photocleavage efficiencies, stepwise yields of the NPPOC and MeNPOC units were determined by comparing the overall yields obtained on various arrays by quantitative hybridisation (see for example Fig. 4): yield (step *n*) = $(y_n) = (I_n/I_{n-1})$, where I_n is the intensity of oligomer of length *n*. Percentage of yield (overall) = $100 \cdot [(y_n) \cdot (y_{n-1}) \cdot (y_{n-2}) \cdot \ldots \cdot (y_2)]$.

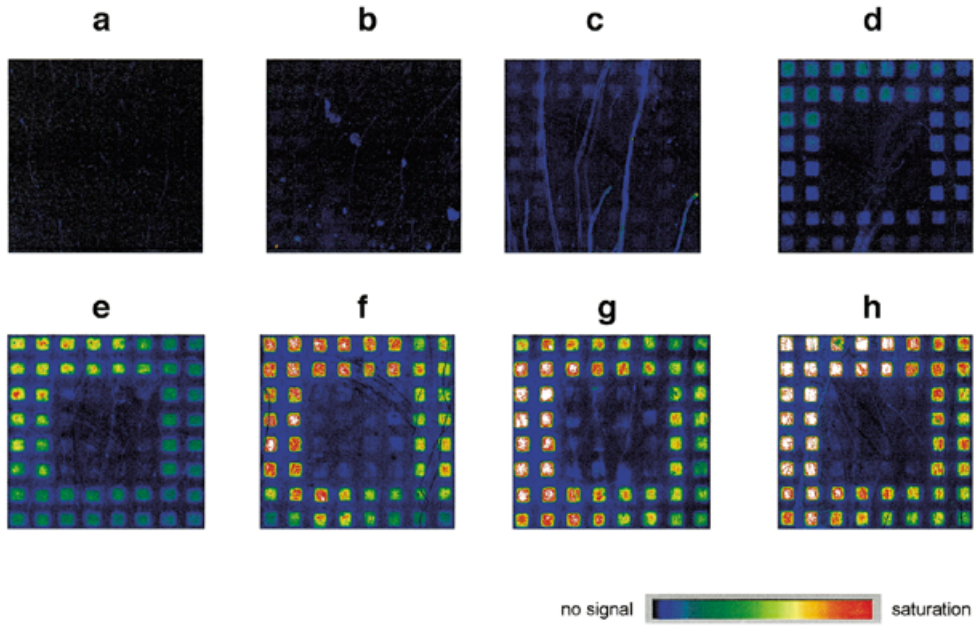

Figure 2. Influence of fluid present during irradiation on synthesis yields with NPPOC nucleosides: (**a**) 10% water in methanol; (**b**) dry (no fluid); (**c**) acetonitrile; (**d**) 0.005 M DBU in acetonitrile; (**e**) 0.05 M DBU in acetonitrile; (**f**) 0.5 M DBU in acetonitrile; (**g**) 0.05 M piperidine in acetonitrile; (**h**) 0.05 M diisopropylethylamine in acetonitrile. During synthesis, the central area on the chip was not illuminated in the ongoing synthesis for control reasons. The sequence on all chips was d(CGCTGGAC), visualised by hybridisation with a labelled 32mer containing the complementary sequence. The bar at the bottom indicates the false colour values.

RESULTS

Instrumental set-up

A 'chip synthesiser' was constructed based on an Eppendorf D200 DNA synthesiser connected to an irradiation unit. The entire synthesis, phosphoramidite coupling as well as removal of the photoprotecting groups, took place in a flow cell in a quasi-continuous manner, with the glass chip actually acting as the cover of the cell. By design, irradiation occurred through the reverse side of the chip. About 10% of the excitation energy was absorbed or reflected by the glass. The only manual operation during the entire process was exchange of the masks placed in a holder directly in front of the flow cell. The relative simple set-up of mask alignment allowed the manufacturing of arrays of up to 400 spots/cm^2. Although of relatively low spot density, such chips were sufficient for monitoring improvements in the chemical aspects of DNA array production.

Rationale of the NPPOC chemistry

Most of the currently used photolabile protecting groups (e.g. MeNPOC) are derivatives of *o*-nitrobenzylalcohol. The NPPOC group is a representative of a family of photolabile protecting groups derived from 2-(2-nitrophenyl)ethylalcohol. They differ especially by an additional methylene moiety on the α-carbon, which is responsible for a completely different photocleavage mechanism. From studies in solution it was concluded that a photoinduced β-elimination process was the favoured pathway for the NPPOC group (12). Therefore, we proposed that the presence of aprotic basic solutions during irradiation might enhance photoremoval of NPPOC moieties. Being aware that the NPPOC moiety is capable of being cleaved off at moderate speed when a strong base is used, conditions had to be defined for practical application, under which not only the deprotection was improved significantly but, simultaneously, base treatment alone would not cause removal of the protecting groups.

Support

Photolithographic array synthesis is routinely carried out on glass, because of its optical features and its inertness to all relevant solvents. However, the strength of oligonucleotide bonding to the support and the relatively low loading capacity are problematical. All experiments described here were done on glass derivatised with a recently described dendrimeric linker system (13). Apart from achieving an increase in loading, if desired, and covalent attachment of the oligonucleotides, both charge and hydrophobicity on the chip surface could be modulated to best meet the specific requirements of the hybridisation experiments.

Synthesis optimisation

For chip production with NPPOC phosphoramidites, irradiation was initially performed in the presence of 10% water in methanol known from in-solution studies (12). However, only DNA arrays of low quality were obtained (Fig. 2a). When irradiation took place in the absence of any fluid, as recommended by Affymetrix for MeNPOC chemistry (9), the syntheses gave comparably unsatisfactory results (Fig. 2b), while MeNPOC phosphoramidites worked as described (8; data not shown).

To test the enhancing effect of adding a base during removal of the NPPOC group, first solutions of DBU in acetonitrile were employed. Indeed, the efficiency of photoremoval was remarkably higher with 0.05 M DBU (Fig. 2e) compared to dry, methanolic or acetonitrile conditions. If the DBU concentration was as low as 0.005 M, only moderate enhancement was observed (Fig. 2d), whereas with 0.5 M DBU partial removal of the NPPOC group by the base alone was detected. On the

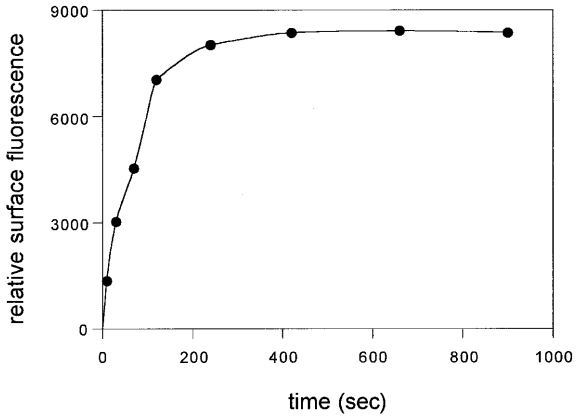

Figure 3. Time dependency of photo-deprotection. The kinetic data were obtained by illuminating a photoreactive surface generated from condensation of a mixture of NPPOC-protected monomers of deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine at 365 nm in 0.05 M DBU and subsequent staining with Cy5-phosphoramidite.

basis of these results, other bases were tested. Piperidine and *N*-diisopropylethylamine especially proved to be very effective promoters in the NPPOC removal process. Best results were achieved with 0.05 M solutions of these two compounds in acetonitrile with 5 min irradiation at 365 nm (25 mW/cm^2). Under such conditions, no removal of the NPPOC moieties by the base alone was detected in the central area of the chips, which by design was not illuminated in the ongoing synthesis in order to allow for an internal control of non-specific deprotection (Fig. 2h).

The half-lives of photochemical deprotection were determined by surface fluorescence (9). The phosphoramidite monomers (dA, dC, dG and dT) were condensed onto a glass surface and irradiated for increasing time intervals in the presence of 0.05 M DBU, removing the photoprotecting groups at defined areas on the surface. Staining these deprotected positions by coupling a Cy5-phosphoramidite and quantification by confocal scanning resulted in kinetic plots as shown in Figure 3. For a critical assessment, the half-life of each of the four monomers was determined individually on separate chips and on one chip. The plots for all four bases essentially looked the same (data not shown) with their half-lives ranging between 50 and 60 s. From this, and for better comparison, the half-life of the mixture of all four monomers was determined to be 55 s. Similar experiments were carried out for the MeNPOC monomers; in our set-up, the average half-life was found to be 53 s. In further experiments, the length of irradiation was 5–6 times the half-life in order to ensure data comparability.

Efficiency comparisons

For a direct comparison of the photochemistries under dry and basic photoremoval conditions, arrays were generated each consisting of oligonucleotides synthesised entirely with 5′-MeNPOC- or 5′-NPPOC-protected phosphoramidites or in part by either chemistry. Measurements were done with different loading capacities of the glass support to avoid a potential influence of oligonucleotide packing. Upon hybridisation with fluorescently labelled target DNA at saturating concentration and under discriminitive hybridisation conditions,

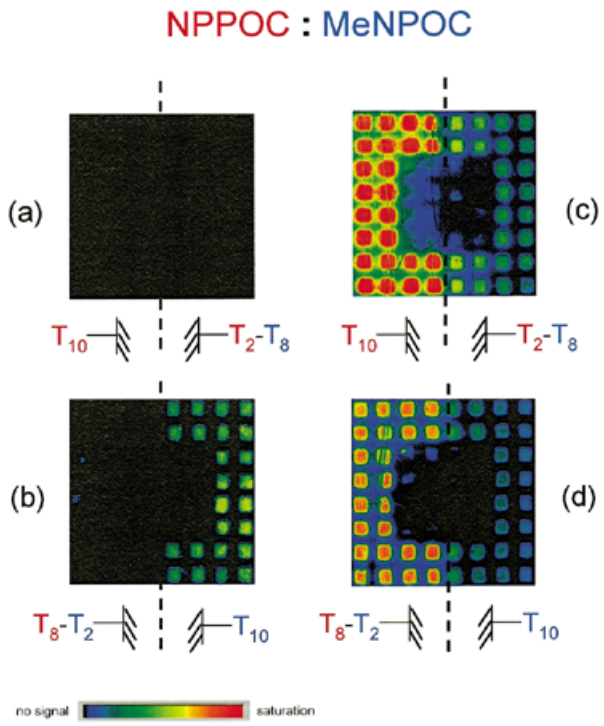

Figure 4. Comparison of photocleavage efficiencies. d(T_{10}) sequences were generated using NPPOC (red) and MeNPOC (blue) phosphoramidites in ratios as depicted. Irradiation conditions: (**a** and **b**) NPPOC (dry), MeNPOC (dry); (**c** and **d**) NPPOC (0.05 M piperidine in acetonitrile), MeNPOC (dry). Signals were obtained upon hybridisation of 5′-Cy5-labelled d(A_{16}). The bar at the bottom indicates the false colour values.

the difference in signal intensities directly reflected the proportional difference in photocleavage efficiency (data not presented). Under dry conditions, as expected, signals were stronger when an oligomer contained more MeNPOC- than NPPOC-derived units (Fig. 4a and b); best yields were found if only MeNPOC phosphoramidites were used (Fig. 4b). However, comparing yields with NPPOC monomers obtained under basic conditions (0.05 M piperidine) with the yields of MeNPOC units under dry conditions, the situation was very different in as much as the more units of NPPOC-protected phosphoramidites were used, the higher the yields became (Fig. 4c and d). Clearly, the overall best results were obtained with only NPPOC chemistry being employed (Fig. 4c). Using arrays produced under the respective optimal synthesis conditions, for MeNPOC phosphoramidites a mean value of 88% of the efficiency per synthesis cycle was observed compared to the NPPOC derivatives. Thus, the eventual overall yield for a typical 20mer built with MeNPOC chemistry is only 8% of that of the same synthesis employing NPPOC chemistry.

Besides polynucleotide sequences, oligomers of mixed nucleotide content were synthesised (see for example Fig. 5). In such experiments, the difference in yield between the two chemistries was even more pronounced than with polynucleotide sequences, probably because of the strict discrimination for full-match hybridisation only, which is difficult to achieve with polynucleotide sequences.

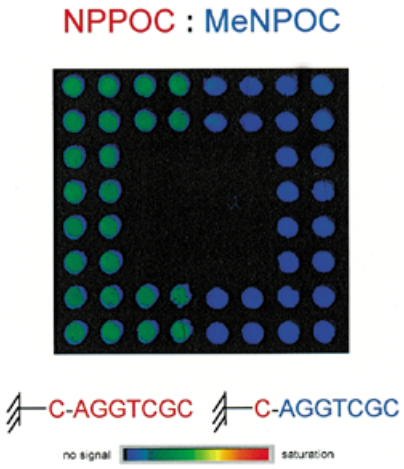

Figure 5. Comparison of oligonucleotide synthesis. d(CGCTGGAC) sequences were generated using NPPOC (red) and MeNPOC (blue) phosphoramidites using the optimal conditions for the respective chemistry. Upon hybridisation of 5′-Cy5-labelled complementary oligonucleotide, signal intensities were scored. The bar at the bottom indicates the false colour values.

The essentially quantitative yield of NPPOC-based on-chip synthesis could also be confirmed by direct visualisation, covalently attaching a fluorescent label at the 5′-end of the oligomers subsequent to photolithographic synthesis (9). By this procedure, only the respective full-length molecules were labelled. On arrays containing sequences of a length between monomer and decamer (Fig. 6a), half the oligomers were permanently Cy5-labelled after synthesis (rows B and D); the other half remained unlabelled (rows A and C). Permanent fluorescence intensities were identical irrespective of the number of cycles. Subsequent hybridisation of the arrays with a Cy3-labelled target DNA, however, resulted in the expected hybridisation pattern, increasing with the length of the oligomers.

Potentially, in such an assay any previously failed deprotection leaving protected molecules could be deprotected in a succeeding step and would result in internally deleted molecules, still capable of incorporating the fluorophore. However, the efficiency of photoremoval not only determines the yields of synthesis but also, and equally importantly, the amount of failure sequences produced. Therefore, by improving the efficiency of photoremoval with NPPOC chemistry, the risk of contamination with such false sequences is concomitantly reduced. From the results of quantitative hybridisation and direct visualisation, no such contamination effect was apparent. Also, even prolonged treatment under the basic conditions alone did not result in any detectable incorporation of fluorescent dye (data not presented), thus indicating that latent deprotection without light excitation was well below the detection level.

Quality assay

For routine quality assessment of DNA chip production, hybridisation assays are inadequate, since only few sequences on a chip can be checked quantitatively with the relevant complementary sequence, and only if the chip is reusable in the first place. Covalently adding a fluorescent marker as described above, on the other hand, assays all oligonucleotides, but influences the hybridisation because of steric and other effects of the dye molecule. Therefore, a removable fluorescent tag had to be used instead. It was added by standard phosphoramidite chemistry as the last step of oligonucleotide synthesis (Fig. 7), consisting of either a dansyl or a Cy5 dye attached to a phosphoramidite building block by a base-labile linker. To check the success of synthesis, the tag was condensed to all full-length molecules only. After recording a signal from each individual spot by scanning (Fig. 7a), the chip was treated with ammonia, concomitantly removing the tag and the base- and phosphate-protecting groups (Fig. 7b). A phosphate unit at the 5′-end of each oligomer was left, which does not interfere with hybridisation (Fig. 7c).

DISCUSSION

By a combination of high yielding chemistry, quality confirmation and documentation of all individual oligonucleotide syntheses, a well-defined system has been assembled for improved oligomer chip analysis. The quantitative synthesis yield affects sensitivity and especially the dynamic range, the latter being a particularly important parameter in quantification. Experimental significance is increased substantially because of improved comparability of the results originating from a notable reduction

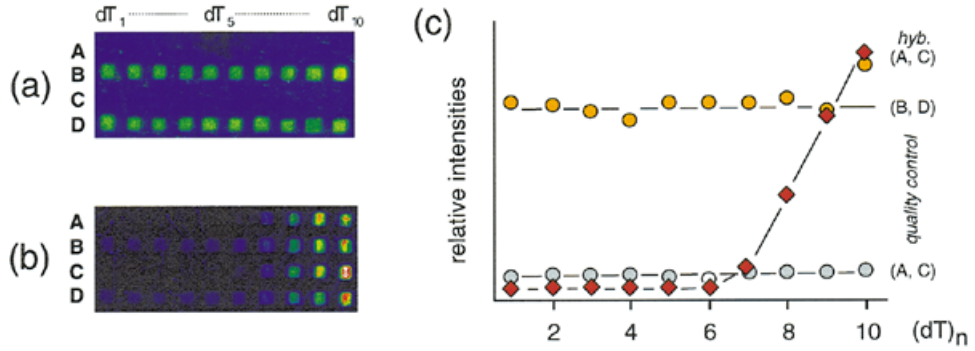

Figure 6. Direct visualisation of synthesis yields. Arrays were synthesised containing, from left to right, monomers to decamers; exemplary results with oligo(dT) are shown. All full-length sequences were permanently Cy5-labelled at their 5′-terminus in rows B and D while they remained unlabelled in rows A and C. Left, the actual images are shown; right, quantification of the signal intensities. (**a**) DNA array after synthesis, scanned in the Cy5-mode. (**b**) DNA array after hybridisation with 5′-Cy3-d(A_{16}); a small background signal from the Cy5-label can be seen in rows B and D, although scanning was performed under conditions optimal for Cy3 detection; false colouring was as above. (**c**) Circles indicate the Cy5-signal in rows B and D and the background signal in rows A and C, respectively; rectangles show the Cy3-intensities obtained in rows A and C upon hybridisation.

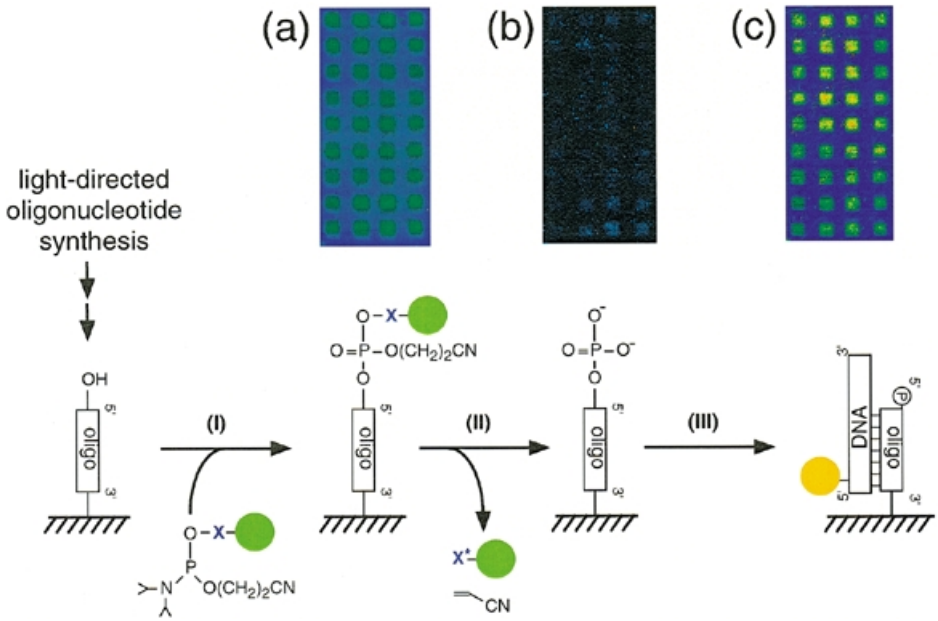

Figure 7. On-chip quality assay. A removable 5′-tag labelled with Cy5 was attached as the last synthesis step (**a**). After quantification, the tag was removed by basic treatment (**b**). Subsequently, the array was subjected to hybridisation with a Cy5-labelled DNA (**c**). False colouring was as shown above. **X** designates a cleavable linker unit.

in chip-caused data variability: reuse of the chips permits the study of several targets under really identical experimental conditions; if different chips are being used, all data can be corrected according to the results of the quality assessment of the individual array positions. Apart from significant improvements in existing applications, new avenues of analysis are opened up. For sequencing by hybridisation with a comprehensive set of oligonucleotides, for example, or any other use which requires absolute mismatch discrimination, the oligomer quality reported here is a prerequisite and normalisation across each data point is essential to achieve distinction.

It is somewhat surprising that NPPOC is photoreactive with decent quantum efficiencies despite its low absorption at the long wavelength used, out-performing the much more absorptive MeNPOC group. A description of the exact cleavage mechanism still awaits clarification.

Use of the NPPOC group has also been extended successfully to the four ribonucleotides, although exact measurements of performance are not finalised. Peptide nucleic acids would also be an interesting substrate, because of their distinct characteristics of interacting with nucleic acids. For many applications, from approaches to highly parallel DNA sequencing (14) to the creation of double-stranded DNA microarrays (15), the availability of a free 3′-terminus and, hence, a reverse direction of oligonucleotide synthesis, would be advantageous. The chemistry described here should also be applicable to this end. With these additional features, photolithographically produced oligomer chips would become an even more versatile tool covering many, if not all, aspects of chip-based analysis procedures.

ACKNOWLEDGEMENTS

We are grateful to Achim Stephan for skilful technical support and Stefan Matysiak for helpful discussions. We thank Wolfgang Pfleiderer (University of Konstanz) and Heiner Giegrich (NIGU-Chemie) for synthesis of the NPPOC and MeNPOC phosphoramidites and discussions. This work was funded by the German Science Ministry (BMBF) under contract 0311663A.

REFERENCES

1. The Chipping Forecast (1999) *Nature Genet.*, **21** (suppl.).
2. Cho,R.J., Campell,M.J., Winzeler,E.A., Steinmetz,L., Conway,A., Wodicka,L., Wolfsberg,T.G., Gabrielian,A.E., Landsman,D., Lockhart,D.J. and Davis,R.W. (1998) *Mol. Cell*, **2**, 65–73.
3. Wang,D.G., Fan,J.B., Siao,C.J., Berno,A., Young,P., Sapolsky,R., Ghandour,G., Perkins,N., Winchester,E., Spencer,J., Kruglyak,L., Stein,L., Hsie,L., Topaloglou,T., Hubbell,E., Robinson,E., Mittmann,M., Morris,M.S., Shen,N., Kilburn,D., Rioux,J., Nusbaum,C., Rozen,S., Hudson,T.J. and Lander,E.S. (1998) *Science*, **280**, 1077–1082.
4. Ahrendt,S.A., Halachmi,S., Chow,J.T., Wu,L., Halachmi,N., Yang,S.C., Wehage,S., Jen,J. and Sidransky,D. (1999) *Proc. Natl Acad. Sci. USA*, **96**, 7382–7387.
5. Winzeler,E.A., Shoemaker,D.D., Astromoff,A., Liang,H., Anderson,K., Andre,B., Bangham,R., Benito,R., Boeke,J.D., Bussey,H. *et al.* (1999) *Science*, **285**, 901–906.
6. Fodor,S.P.A., Rava,R.P., Huang,X.C., Pease,A.C., Holmes,C.P. and Adams,C.L. (1993) *Nature*, **364**, 555–556.
7. Pease,A.C., Solas,D., Sullivan,E.J., Cronin,M.T., Holmes,C.P. and Fodor,S.P.A. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 5022–5026.
8. Pirrung,M.C. and Bradley,J.-C. (1995) *J. Org. Chem.*, **60**, 6270–6276.
9. McGall,G.H., Barone,A.D., Diggelmann,M., Fodor,S.P.A., Gentalen,E. and Ngo,N. (1997) *J. Org. Chem.*, **119**, 5081–5090.
10. Pirrung,M.C., Fallon,L. and McGall,G. (1998) *J. Org. Chem.*, **63**, 241–246.
11. Hasan,A., Stengele,K.P., Giegrich,H., Cornwell,P., Isham,K.R., Sachleben,R. and Pfleiderer,W. (1997) *Tetrahedron*, **53**, 4247–4264.
12. Giegrich,H., Eisele-Bühler,S., Hermann,C., Kwasyuk,E., Charubala,R. and Pfleiderer,W. (1998) *Nucl. Nucl.*, **17**, 1987–1996.
13. Beier,M. and Hoheisel,J.D. (1999) *Nucleic Acids Res.*, **27**, 1970–1977.
14. Ronaghi,M., Uhlen,M. and Nyren,P.A. (1998) *Science*, **281**, 363–365.
15. Bulyk,M.L., Gentalen,E., Lockhart,D.J. and Church,G.M. (1999) *Nature Biotechnol.*, **17**, 573–577.