

BTA, a novel reagent for DNA attachment on glass and efficient generation of solid-phase amplified DNA colonies

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ABSTRACT

The tricarboxylate reagent benzene-1,3,5-triacetic acid (BTA) was used to attach 5'-aminated DNA primers and templates on an aminosilanized glass surface for subsequent generation of DNA colonies by *in situ* solid-phase amplification. We have characterized the derivatized surfaces for the chemical attachment of oligonucleotides and evaluate the properties relevant for the amplification process: surface density, thermal stability towards thermocycling, functionalization reproducibility and storage stability. The derivatization process, first developed for glass slides, was then adapted to micro-fabricated glass channels containing integrated fluidic connections. This implementation resulted in an important reduction of reaction times, consumption of reagents and process automation. Innovative analytical methods for the characterization of attached DNA were developed for assessing the surface immobilized DNA content after amplification. The results obtained showed that the BTA chemistry is compatible and suitable for forming highly dense arrays of DNA colonies with optimal surface coverage of about 10 million colonies/cm² from the amplification of initial single-template DNA molecules immobilized. We also demonstrate that the dsDNA colonies generated can be quantitatively processed *in situ* by restriction enzymes digestion. DNA colonies generated using the BTA reagent can be used for further sequence analysis in an unprecedented parallel fashion for low-cost genomic studies.

INTRODUCTION

The goal of a cost-effective approach to whole-genome resequencing is the impetus for current research efforts that are focused on the development of novel, highly efficient DNA sequencing methods (1). Next-generation technologies for low-cost DNA sequencing will be widely applicable, and will have a strong impact on biomedical research. An important example is the sequencing of individual genomes as a component of predictive and preventive medicine, and for hypothesis testing toward the discovery of genotype-phenotype associations (2-6).

A series of massively parallel DNA sequencing methods have been developed toward the goal of ultra low-cost sequencing (7-11). One of the most promising techniques make use of parallel sequencing through the synthesis of very dense DNA colony arrays, generated by solid-phase amplification of surface-attached single-template molecules (12-14).

A suitable approach for performing the *in situ* amplification of target DNA templates (generation of DNA colonies) consists of the initial attachment of amplification primers by 5' termini, which allows the free 3' ends to prime DNA synthesis through DNA templates that hybridize to the surface-bound primers. With this method, DNA can be amplified by two mechanisms: (i) interfacial amplification (priming step) followed by surface amplification (12), or (ii) amplification of primers and target templates after simultaneous attachment to the surface by suitable functional groups at the 5' ends (co-grafting) described in the present manuscript.

For the two experimental approaches of priming and co-grafting the attached DNA must satisfy the requirements imposed by the subsequent solid-phase amplification by thermocycling. First, the primers (or both primers and template for the co-grafting approach) must be surface-bound by a 5' end-specific linkage to ensure that the primer can participate in polymerase-mediated elongation during the solid-phase

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PCR process. Second, the surface density of attached oligonucleotide primers must exceed a critical value for efficient amplification that permits detection by fluorescence in subsequent sequencing by primer extension or hybridization assays. Third, the covalent linkage between the starting DNA and the surface should be sufficiently stable and resistant to the repeated heating and cooling cycles of the PCR amplification procedure. Therefore, solid-phase DNA amplification requires a well-characterized and reproducible DNA attachment chemistry for strict control over the most critical parameters, such as the ratio of attached primer and template DNA, and the specific conditions of thermocycling. The template/primer ratio defines the surface density of single-molecule templates, and thus the final surface coverage of DNA colonies generated after amplification. The nature and number of thermal cycles together with the thermal stability of the surface defines the net efficiency of amplification that is, the average number of copies of the original single-molecule template that composes each colony.

Several chemical strategies have been described for the attachment of DNA on solid surfaces, such as beads (15) or glass primarily for the production of oligonucleotide arrays (16–19). In addition, chemically-modified glass has been exploited as a substrate in solid-phase DNA template amplification (12), solid-phase mRNA transcription into cDNA and its storage (the so-called Expression Snapshot™ mRNA Archiving), solid-phase mRNA amplification (<http://www.lindenbioscience.com>), primer amplification (20,21) and in solid-phase DNA microsequencing (22). Each of these applications may require specific control over the reactivity of the chemically-modified surface toward the biomolecule to be attached.

Previously, we have compared thiol-based chemistries using heterobifunctional cross-linkers for their applicability in solid-phase DNA amplification (12). However, the selected reagent *m*-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sulfo-MBS) produces low surface concentrations of grafted primers and does not allow for efficient DNA bridging which results in low yields of solid-phase-amplified DNA. Another disadvantage of this reported method arises from the use of oligonucleotides that require an extra chemical (thiol) modification during synthesis. This could be an added source of experimental variability caused by oxidation during storage, which could decrease the reproducibility of colony formation.

To design a more efficient chemistry compatible with DNA surface amplification, we established two criteria for the selection of chemical cross-linkers; the use of commercially available and stable 5'-aminated oligonucleotides, and the use of aminosilanized surfaces. With two primary amino functionalities to link together, we explored polycarboxylate linkers for the generation of two stable amide bonds where their formation can be catalyzed by common bulk catalysts.

In the work reported herein we have exploited bulk catalyst mediated covalent attachment of tricarboxylic acid (TCA) to aminosiloxane-modified glass surfaces, followed by carbodiimide-catalyzed immobilization of amine-containing DNA to the TCA surface. The aromatic trifunctional compound used was benzene-1,3,5-triacetic acid (BTA) but also trimesic acid (TMA) has been tested in our lab. We have chosen these aromatic trifunctional linkers over aliphatic

bi and TCAs because of the following reasons: aliphatic acids having two or three carboxylic groups at the extremity of the aliphatic chain would be more flexible than aromatic linkers and as a result, their carboxylic acids, once activated, could react with the amine-terminated glass surface. This could introduce a certain degree of hydrophobicity to the surface and decrease the amount of free carboxylic acid groups available for biomolecule attachment.

The BTA chemistry relies on bulk catalysis for both the glass carboxylation step as well as the covalent coupling of the aminoalkyl-substituted DNA to carboxyl-terminated surface. This, in turn, results in a more robust chemistry in comparison with classical methods, which rely exclusively on the use of activated bifunctional linkers.

The first part of this report focuses on the characterization of BTA surfaces for the chemical attachment of oligonucleotides. We evaluate the properties relevant to the *in situ* amplification process: surface density levels attained, specificity of 5' end attachment, thermal stability of the attached DNA under thermocycling conditions, reproducibility of glass functionalization, and long-term storage stability of BTA-derived glass surfaces. In the second part, we describe quality control (QC) methods developed for content analysis of DNA bound to the surface of general applicability. We exemplify one of our analytical methods through the assessment of DNA colonies that were generated with the BTA cross-linking reagent. In particular, these QC methods were applied to dsDNA colonies that had been digested *in situ* with a type II_s-restriction enzyme.

MATERIALS AND METHODS

Chemicals were supplied by Aldrich, Fluka and Riedel de Haën and used without additional purification. Anhydrous acetone, anhydrous dimethylformamide (DMF), absolute ethanol and acetonitrile [high-performance liquid chromatography (HPLC) gradient grade] were from SdS. Biological buffers were prepared in house; 20× SSC buffer contains 3 M NaCl and 0.3 M sodium citrate. TE buffer is made from Tris base at pH 8 (0.1 M) and EDTA (0.01 M), TMN buffer is made from Tris-HCl (pH 7.5) (0.01 M), NaCl (0.05 M) and MgCl₂ (0.02 M). *N,N*-diisopropylethylamine (DIEA, peptide synthesis grade) was from Perkin Elmer. The heterobifunctional cross-linker succinimidyl 3-[2-pyridyl]dithio]propionate (SPDP) was purchased from Pierce. 3-Aminopropyltriethoxysilane (APTS) was purchased from Aldrich. Aliquots of 2 ml of the silanization reagent were prepared from the initial batch and stored in vials closed with a rubber septum. Once opened, an aliquot was used within a week, and each time restored under an argon atmosphere. 1-4-dithio-DL-threitol (DTT), *N*-hydroxysuccinimide (NHS), *N,N'*-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), 1-methylimidazole, BTA and 4-fluoro-7-nitrobenzofurazan (NBD-F) were purchased from Fluka. *m*-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (sulfo-MBS) was purchased from Pierce. Texas Red®-X and sulfocyanine dye Cy 5.0 succinimidyl esters were prepared by using published procedures (23,24). Cyanine Cy 5.0 labeled aminopropargyl dideoxynucleotides (ddNTP-Cy 5.0) were prepared according to reported

methods (25). All reactions were run in oven-dried glassware (flasks and silanization tanks). Oligonucleotides were purchased from Eurogentec S.A. (Brussels, Belgium). *Taq* polymerase was supplied from Promega, Terminal Transferase, BbvI and bacteriophage lambda DNA were from New England Biolabs. Microscope glass slides ($76 \times 26 \times 1 \text{ mm}^3$, Knittel, Merck, ABS Postfach, Germany) were obtained from commercial sources. Microfabricated chips were obtained from Micronit (Twente, NL) or IMT (Neuchatel, Switzerland) according to design specifications described below. Electropherograms were obtained using a Sequencer Long Read Tower System from Visible Genetics. Cy5.5-labeled DNA markers and Cy5-labeled ddNTPs were purchased from Amersham. Fluorescence measurements were performed using an inverted fluorescence microscope equipped with an arc mercury lamp (Axiovert 200 + HBO 100W/2, Carl Zeiss) and coupled to a CCD camera ORCA ER from Hamamatsu. A main concern to quantitative analysis of fluorescence intensities is the fluorophore bleaching. The focus position was approached under regular top light. This method allowed reaching the final focus within a few seconds, minimizing the fluorophore bleaching. Moreover, data analysis was always processed from at least five images per sample (slide or channel). Fluorescent signals were measured by integration of the signal in an image within house developed software. HPLC analyses were performed on an Agilent model 1100 analytical HPLC unit equipped with a Waters XTerra MS C18 ($2.5 \mu\text{m}$, $2.1 \times 50 \text{ mm}$) column. Purifications of modified oligonucleotides were performed on a Waters model Breeze preparative HPLC unit. All electrospray ionization (ESI) mass spectra were recorded on a Quattro Micro QAA118 spectrometer from Micromass.

Synthetic oligonucleotides

Synthetic oligonucleotides were designed as follows: Primer-P1: CACCAACCCAAACCAACCCAAACC; Primer-P2: GAGGAAAGGGAAGGGAAGGAAGG; Reverse-P1: GGTGGGTTGGTTGGGTTGGTG; Reverse-P2: CCTTCCTTCCCTTCCCTTTCCTC. These sequences have been reported previously for their use in solid-phase DNA amplification (12). The forward primers were used in the present work with or without an extra 10 T sequence in the 5' position as indicated in the text. The modifications of oligonucleotides at 5' ends were performed in our lab and are described below. The purity level of all oligonucleotides was systematically checked by HPLC and ES-MS before use.

DNA templates

A fragment from bacteriophage lambda DNA (ci857ind 1 Sam 7) containing a single recognition site for the restriction enzyme BbvI was generated by PCR using amplification primers that contained the sequences P1 and P2 described below. The resulting template (T1) flanked by p1, p2 sequences is 347 base pairs long and contains a unique BbvI recognition site, GCAGC, at position 288–292.

The following synthetic 80mer oligonucleotide of sequence: GAGGAAAGGGAAGGGAAGGGAAGGATCCTTCTGCA-GGTCATCGGCATGTAGAATTCGGTTGGGTTGGTTGGGTTGGTG has been used as a synthetic template (T2) bearing modifications at its 5' end, performed according to

the method described below, for introducing a disulfide linker moiety and a free primary amino functionality resulting in a 5'-amino-SS-T2 template.

General synthesis of cleavable disulfide primers

Lyophilized 5'-NH₂ oligonucleotide (15 nmol) was dissolved in 0.1 M sodium bicarbonate buffer (pH 8.8) (150 μl). A total of 60 μl of solution of SPDP in dry acetonitrile (1 mg/100 μl , 130 equiv.) were added and the resulting mixture was periodically vortexed for 1 h at room temperature. Thereafter, 100 μl of solution of cysteamine (1.5 mg, 19.45 μmol , 1300 equiv.) in 0.1 M Tris-HCl buffer (pH 7.5) were added and the resulting mixture was again periodically vortexed for 2 h at room temperature. After dilution with 0.1 M triethylammonium acetate (pH 7.0) (TEAA buffer, 1.5 ml), the cleavable disulfide primer is purified by reversed-phase HPLC by using the following conditions: (C8, Zorbax Eclipse, 5 μ , 4.6×150) with acetonitrile and TEAA buffer [0.1 M and (pH 7.0) [100% TEAA (5 min), then linear gradient from 0 to 5% of acetonitrile (5 min) and 5 to 50% (150 min)] at a flow rate of 1 ml/min. Dual ultraviolet (UV) detection was achieved at 214 and 260 nm. To load the oligonucleotide properly onto the column, it is essential to perform three individual injections before running the gradient. The product-containing fractions are three times lyophilized to remove significant amounts of TEAA salts. Stock solutions were prepared in HPLC water and concentration was determined by UV quantification at $\lambda = 260 \text{ nm}$ by using the ϵ value of the corresponding 5'-NH₂ oligonucleotide (yield 20–70%).

General method for the fluorescent labeling of synthetic oligonucleotides with Texas Red[®]-X succinimidyl ester

Lyophilized 5'-NH₂ oligonucleotide ($\sim 20 \text{ nmol}$) was dissolved in 0.1 M sodium bicarbonate buffer (pH 8.8, 30 μl) and DMF (10 μl). The solution of Texas Red[®]-X succinimidyl ester in dry DMF (0.1 mg in 10 μl , 122 nmol, 6 equiv.) was added and the resulting mixture was protected from the light and periodically vortexed for 1 h at room temperature [after several minutes, further amount of 0.1 M sodium bicarbonate buffer (40 μl) or triethylamine was added to reach again a pH of 9]. After dilution with TEAA buffer (1 ml), the fluorescent primer was purified by reversed-phase HPLC by using the following conditions: (C8, Zorbax Eclipse, 5 μ , $4.6 \times 150 \text{ mm}$) with acetonitrile and TEAA (0.1 M, pH 7.0) [100% TEAA (1 min), then non-linear gradient from 0 to 50% acetonitrile from 0 to 20% (5 min), 20 to 25% (10 min) and 25 to 50% (100 min)] at a flow rate of 1 ml/min. Dual UV detection was achieved at 595 and 260 nm. The product-containing fractions were three times lyophilized to remove significant amounts of TEAA salts. Stock solutions were prepared in HPLC water and concentration was determined by UV quantification at $\lambda \sim 592 \text{ nm}$ by using the $\epsilon = 85\,000$ value (yield 20–95%).

General method for the fluorescent labeling of synthetic oligonucleotides with sulfocyanine dye Cy 5.0 succinimidyl ester

Lyophilized 5'-NH₂ oligonucleotide (10–15 nmol) was dissolved in 0.1 M sodium bicarbonate buffer (pH 8.8, 65 μl). The solution of Cy 5.0 succinimidyl ester in dry DMF (0.24 mg

in 10 μ l, 370 nmol, 25–35 equiv.) was added and the resulting mixture was protected from the light and periodically vortexed for 1 h at room temperature. After dilution with 0.1 M aqueous tetramethylammonium chloride buffer (TMA-Cl, 0.1 M, 1.0 ml), the crude reaction mixture was firstly purified by anion-exchange chromatography by using the following conditions: chromatography column (Econo-Pac[®] Disposable from Bio-Rad) filled with a solution of DEAE-Sephadex A-25 (40–120 μ m, from Aldrich 0.75 g and 1.5×3.0 cm bed) in methanol and equilibrated with (i) deionized water (50 ml), (ii) 1.0 M aqueous TMA-Cl (100 ml) and (iii) 0.1 M aqueous TMA-Cl (100 ml). The column was eluted with a linear gradient of aqueous TMA-Cl from 0.1 to 1.0 M. The product-containing fractions were lyophilized resulting in a blue oily residue which was redissolved in TEAA buffer and purified by reversed-phase HPLC by using the following conditions: (MS C18, Waters XTerra, 5 μ , 7.8×100 mm) with acetonitrile and TEAA (0.1 M, pH 7.0) [100% TEAA (15 min), then linear gradient from 0 to 50% of acetonitrile (50 min)] at a flow rate of 3 ml/min. Dual UV detection was achieved at 650 and 260 nm. The product-containing fractions were three times lyophilized to remove significant amounts of TEAA salts. Stock solutions were prepared in HPLC water and concentration was determined by UV quantification at $\lambda = 648.5$ nm by using the $\epsilon = 250\,000$ value (yield 20–70%).

The structure of all cleavable disulfide or fluorescent primers was confirmed by ESI mass spectrometry and the purity was checked by reversed-phase HPLC.

Synthesis of ethylenediamine-NBD (NBD-NH₂)

Ethylenediamine (206 μ l, 3.07 mmol) was mixed in a 10 ml flask with 0.3 ml of dry DMF at 4°C. A solution containing 5.63 mg of NBD-F (0.03 mmol) in 0.1 ml of dry DMF was added dropwise to the ethylenediamine solution over 5 min. The resulting reaction mixture was stirred at room temperature, in the absence of light, for 2 h. Thereafter, solvents were removed under reduced pressure. The resulting orange oily residue was purified by chromatography on a silica gel (10 g) column with a step gradient of methanol (0 to 30%) in dichloromethane as the mobile phase. The appropriate fractions were pooled and then concentrated to dryness giving 2.56 mg of ethylenediamine-NBD as an orange solid (yield 37%). TLC (CH₂Cl₂/CH₃OH 80/20 v/v) $R_f = 0.33$ and 1.00 (starting material, NBD-F); MS (ESI⁺) m/z 245.64 (M+Na)⁺, 223.71 (M+H)⁺ (calculated from the Isis Draw software : 223.19 for C₈H₉N₅O₃).

The synthesis of succinimidyl ester of BTA

We have elaborated experimental protocol for the conversion of 32 aminosilanized 'ATS slides' (respectively 16 'ATS channels') to 'BTA slides' (respectively 'BTA channels') carboxyl-terminated glass. This procedure can be further scaled up for desired number of samples. Starting reagents are weighed in Eppendorf tubes. Typical amounts are 60.5 mg of BTA (tube labeled 'BTA'), 99.4 mg of NHS (tube labeled) and 149.6 mg of *N,N'*-dicyclohexylcarbodiimide (tube labeled 'DCC'). Subsequently, 400 μ l DMF were pipetted respectively in the BTA and the 'NHS' tube; and 200 μ l DMF were pipetted in the 'DCC' tube. Solids were solubilized

by vortexing each tube. A small magnetic bar was placed in a 10 ml glass flask fixed above the magnetic bar stirrer. The stirring was set at a moderate speed. The content of the 'BTA', 'NHS' and 'DCC' tubes were then transferred by pipetting in the glass flask in the following order: first BTA solution, then NHS solution and finally DCC solution. This latter step was executed quickly. The glass flask was then closed immediately and kept under dry and controlled atmosphere (rubber septum equipped with a needle for the N₂ inlet and outlet). Active ester formation should be accompanied by the formation of a precipitate (disubstituted urea formed from DCC), perceptible within 5 min following the addition of all the components in the reaction mixture. The reaction was left to proceed under a constant stirring for 2 h. Solution as well as solid material were recovered from the glass flask and centrifuged in a Eppendorf 5415 centrifuge at 10 000 r.p.m. (9.3 g) for 5 min; this allowed the separation of the liquid phase containing the active ester (clear yellow solution) and the solid-phase (disubstituted urea). In the meantime, 965.8 μ l DMF were pipetted in the Eppendorf tube labeled 'DIEA' and 34.2 μ l *N*-ethyl-diisopropylamine were rapidly added. The active ester is taken from the centrifuged tube, avoiding pipetting solid precipitate particles, and mixed with an equal volume of DIEA solution in an Eppendorf tube. The solution is shortly vortexed and used straight after. The reaction was checked to completion by ESI mass spectrometry (recorded in the negative mode). Two major peaks assigned to the di- and tri-succinimidyl ester of BTA (calculated from the Isis Draw software: 446.37 for C₂₀H₁₈N₂O₁₀ and 543.45 for C₂₄H₂₁N₃O₁₂) were observed. Non-quantitative conversion of BTA into tri-succinimidyl ester can be explained by partial co-precipitation of these hydrophobic esters with *N,N'*-dicyclohexylurea (DCU).

Glass slide surface activation and aminosilanization

The protocol for glass activation described here is similar to the previously reported method (12), except that the time of glass slide treatment in 1 M HCl has been reduced from 12 to 1 h and other minor modifications. Glass slides were soaked in a 1 M NaOH solution, in the presence of a detergent (Hellmanex II, 0.1%) for 1 h, rinsed with water for 5 min, immersed in a 1 M HCl solution for 1 h, rinsed again in water for 5 min and treated for 1 h in sulfuric acid solution (H₂SO₄/H₂O, 1/1, v/v). Acid-treated slides were submitted to extensive washes in water followed by rinsing in absolute ethanol, flushed under a stream of nitrogen, dried in the desiccator for 20 min (under vacuum) and stored under dry vacuum for further use. Silanization of a batch composed of 8 hydroxylated glass slides prepared as described above was done in a silanization tank containing a solution of 3-aminopropyltriethoxysilane (APTS) in dry acetone (Acetone/APTS, 95/5, v/v) for 1 h at room temperature. Aminosilanized slides (ATS) were then washed three times with 100 ml of anhydrous acetone (5 min per washing, mild shaking), rinsed with ethanol, dried under a stream of N₂ and stored under dry vacuum before further use.

Carboxylation of ATS glass slides

Sixteen 'ATS' slides were disposed on a glass support and 100 μ l of the active ester/DIEA solution was pipetted on each

glass slide and covered immediately with another ATS slide (face to face with aminosilanized side). Surface carboxylation reaction was typically left to proceed for 3 h. Slides are manipulated using tweezers.

BTA-derivatized glass slides were removed from the glass support and washed with DMF and with ethanol solutions. The BTA slides were dried under a stream of nitrogen and kept under dry vacuum before further use. Before their use, NHS was released from the BTA glass surface by hydrolysis in the hydrogen carbonate solution. Typically, using a glass flask filled with 200 ml of 5% NaHCO₃ (pH 8.80). The hydrolysis was left to proceed for 15 min. BTA slides were rinsed under running Millipore water for 2 min (using tweezers), rinsed with ethanol, dried under the stream of nitrogen and kept under vacuum before further use. BTA slides stored under vacuum were found to be stable for at least one month.

Staining of the aminosilanized (ATS) slides with NBD-F

This protocol describes the QC procedure that allows to follow batch-to-batch reproducibility of glass aminosilanization based on the reaction of NBD-F with the amine-terminated ATS glass. Fluorescence intensity of NBD-stained ATS slides is expected to correlate with the surface concentration of amino groups on the glass surface.

Eight ATS-silanized glass slides were reacted with a 2.7 mM solution of NBD-F (0.26 mg) and a 5.5 mM solution of DIEA (0.5 μ l) in dry DMF. A volume of 100 μ l of solution was used for two slides placed face to face for 1 h at room temperature in the absence of light. NBD-stained glass slides were then washed successively with DMF and ethanol and dried with a stream of N₂. The fluorescence was measured in the air using the inverted microscope equipped with the 20X objective and Xf43 filter (Omega Optical).

Staining of carboxyl-terminated glass slides with NBD-NH₂

The protocol described below allows staining of eight carboxyl-terminated BTA slides to follow batch-to-batch reproducibility of glass carboxylation. Four BTA slides labeled #1–4 were placed on a clean glass support (17 \times 14 cm glass plate). Two glass cylinders (4 mm in diameter) were placed under the slides at both extremities (in a form of rails) in order to avoid staining of slides on both sides.

NBD-NH₂ (0.34 mg, 1.52 μ mol) was weighed in the dark Eppendorf tube and dissolved in 600 μ l DMF. DIEA (0.78 μ l, 4.71 μ mol) was then added and the resulting solution A was briefly vortexed. Benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP) (0.67 mg, 1.51 μ mol) was weighed in another Eppendorf tube and dissolved by brief vortexing in 200 μ l DMF (solution B). Solutions A and B were then mixed together and shortly vortexed (solution C).

An aliquot of 100 μ l of solution C were applied immediately on slide #1 and spread by placing the slide #2 on top of it. The same procedure was repeated for the slides #3–8. The staining reaction was let to proceed for 60 min in the absence of light. The glass slides were then washed successively with DMF and ethanol, dried with a stream of N₂ and kept under vacuum for at least 20 min (avoid aqueous solutions since NBD fluorescence is strongly quenched in the presence of water).

The fluorescence for NBD-stained slides was taken in the air using the inverted microscope equipped with the 20X objective and Xf43 filter.

Attachment of aminated oligonucleotides to carboxylated slides

5'-Aminated oligonucleotides (C6 linker) were prepared as a 0.1 to 1 μ M solution in 10 mM 1-methyl-imidazole (pH 7.0) containing 10 mM EDC. Frame seal wells (Bioconcept) were fixed on BTA glass for creating compartments that were filled with 25 μ l of the reaction mix (on ice). Glass slides were then incubated at 50°C for 10–60 min in a humid atmosphere to avoid evaporation. The reaction solution was carefully removed with a pipette and each compartment was washed three times with 0.1 \times SSC-0.1% Tween, and three times with 5 \times SSC solutions (each wash for 1 min). The glass slides were gently shaken for 10 s at the end of each wash. Slides were stored in 5 \times SSC at 4°C for further use.

Design and chemical treatments of glass microchannel chips

BTA chemistry was implemented on glass microfabricated channels, and was adapted for process scale-up, while maintaining a closed and clean environment to minimize any contamination risks. The fluidics-integrated chip is suitable for full automation of all the chemical and biochemical steps involved in the colony generation process. In microfluidic devices (defined as devices where at least one dimension is less than 1 mm), the flow is generally laminar (low Reynolds number), making molecular diffusion the dominant mechanism of chemical reactions. Moreover, microfluidic devices show a large surface-to-volume ratio. For these two reasons, the typical kinetic constant of chemical reactions is drastically increased in microfluidic devices compared to macroscopic systems (26). Therefore, the timing of chemical reactions was adapted to the micro-scale of the surface to be derivatized; specifically, a typical 1 h treatment could be reduced to just a few minutes. Detailed procedures concerning each step of the chemical modifications are given in the following paragraphs.

A chip consists of eight channels etched in a glass support, each with dimensions of (l = 20) \times (w = 1) \times (h = 0.1) mm³. Chip inlets and outlets were connected to a main fluidic system through flanged tubing, and fluids were driven with peristaltic pumps as described previously (27).

A thermocycler from MJ-Research was customized and used for all steps where a temperature modulation was required. The chip is installed on a flat heating plate; an aluminum adaptor insures a thermal contact between the chip and the heating plate. Due to thermal losses, a temperature shift exists between the temperature set on the thermocycler and the actual temperature at the channel level. A calibration was performed to determine the temperature offset as a function of the set temperature. The channel temperature is estimated by measuring the front and back sides of the chip with thermocouples assuming a linear temperature gradient across the chip thickness.

The compositions of the solutions used for channel derivatization are identical to what is described in the glass slide treatments sections, only the timing, soaking and rinsing differ.

The solutions are pulled through the channels; the pump flow rate is set on 25 $\mu\text{l}/\text{min}$ and continuous flow, unless otherwise specified.

It has been clearly noticed that introduction of air in the tubing is very harmful to a homogeneous surface derivatization. Particularly, no bubbles must be stuck inside the channels, at any step described below.

The cleaning and hydroxylation consist in the following sequence of solutions: Hellmanex 1% (5 min), NaOH/Hellmanex 1% (5 min), deionized water (3 min); then HCl 1 N (5 min) and sulfuric acid solution (5 min). The channels are then conditioned for the silanization by running ethanol through them (3 min) and acetone (3 min). Channels are then filled with the ATS solution. The pump is stopped for 3 min, restarted for 20 s; this cycling mode is operated during 10 min. Channels are rinsed with acetone (3 min) and ethanol (3 min). At this step, the chip is disconnected from the pump and nitrogen at a pressure of 400 bars is pushed through each channel during 20 s. The chip can be stored under dry vacuum.

The chip is reconnected to the peristaltic pump to perform the carboxylation. BTA solution is freshly prepared as described previously and channels are filled with it. The pump is stopped and operated in cycling mode during 10 min. At the end of the 10 min, the pump is switched back on continuous mode and the rinsing steps are performed: DMF (2 min), ethanol (3 min), deionized water (3 min), NaHCO_3 solution (5 min), deionized water (3 min) and ethanol (3 min). Finally, channels are dried as described previously and can be stored in a desiccator before further use.

Grafting and solid-phase amplification on BTA glass and generation of DNA colonies in channels

The next paragraphs detail the procedure for grafting and PCR amplification. The timings and temperatures are given, taking into account the thermal inertia of the chip mounted on the MJ-Research thermocycler. Solutions are prepared as for glass slides and concentrations given for a standard process.

The pump flow rate is set at 15 $\mu\text{l}/\text{min}$ during the whole grafting process. The grafting solution is freshly prepared at a concentration of 1 μM in primers. It is critical that the solution is introduced into the channels as the temperature is stabilized at 51°C. The channels stay in contact with the grafting solution during 30 min while the temperature remains at 51°C and the pump is run in cycling mode to save grafting solution (flushing 20 s every 3 min). The rinsing with 0.1 \times SSC-0.1% Tween can start when the temperature is stabilized back to 20°C and for 5 min, followed by a 5 min rinse with 5 \times SSC. The chip can be stored at room temperature in 5 \times SSC buffer.

The PCR mix solution contains enzyme buffer (1 \times), betain (1 M), DMSO (1.3%), dNTPs (200 μM), *Taq* polymerase (0.025 U/ μl) and HPLC quality water. The channels are rinsed 5 min with deionized water prior to the amplification process to avoid possible inhibition of the polymerase enzyme due to excessively high saline conditions. The PCR mix is introduced in the channel and the amplification begins denaturing conditions: the temperature is set to 97.5°C and reached at a rate of 0.5°C/s to avoid thermic shock and eventually breaking the chip. The temperature cycling consist in 20 min at 97.5°C (denaturation), 1 min 30 s at 61°C (annealing) and 1 min

30 s at 73°C (extension); this sequence is repeated 40 times. During the whole cycling process, the channels are in static contact with the PCR mix solution, except for the last 20 s of the denaturing step where the pump is run at a rate of 15 $\mu\text{l}/\text{min}$ to bring fresh PCR mix. After the 40 temperature cycles are completed, the temperature is set at 73°C. The rinsing solutions are flushed at a flow rate of 15 $\mu\text{l}/\text{min}$ the pump set back in continuous mode. First rinse is made with 0.1 \times SSC-0.1% Tween and final rinse with 5 \times SSC. The channels may be stored in this buffer.

Visualization of dsDNA colonies by using Sybr-Green I

Amplified DNA colonies were stained with a 10 000-fold diluted solution of Sybr-green I in TE for 5 min at RT. Images were taken using the inverted microscope using a filter set xf22 (Omega Optical) with 1 s irradiation time.

RESULTS AND DISCUSSION

Analytical characterization of functionalized glass slides

The formation of an aminosiloxane film on the hydroxy group-terminated glass surface and its subsequent conversion from the amine-functionalized ATS glass into a carboxyl-terminated surface with BTA(NHS)₂ is shown in Figure 1A. A coupling protocol similar to that described for BTA also applies for another structurally similar molecule, TMA, (see Materials and Methods section). The efficiency of the carboxylation of glass surfaces with BTA and TMA was determined by using two methods: a direct-labeling of the glass surface with a functionalized fluorescent reporter group (slide staining), and an indirect method that involves the immobilization of 5' end-aminated oligonucleotide primers followed by the hybridization of a complementary oligonucleotide probe labeled with a fluorescent molecule.

The staining of glass slides with two fluorescent NBD derivatives is shown schematically in Figure 1B and C. Surfaces were stained with NBD-F and NBD-NH₂ to compare the specific and non-specific attachment yields, respectively, for the same fluorophore on the ATS surface. Figure 2A shows that the amino groups of ATS were stained specifically by the NBD-F reagent, whereas staining by NBD-NH₂ gave much lower fluorescent signal, as expected for the negative control. In the case of BTA surface, carboxyl groups were activated with the peptide coupling phosphonium reagent BOP [for a recent review on peptide coupling reagents, (28)] and allowed to react with NBD-NH₂ in the presence of the tertiary amine *N,N*-diisopropylethylamine (DIEA), and gave very good yields. Again, only very low background fluorescence for non-specific staining was observed.

We found that very reproducible fluorescence recovery was possible with N₂-dried slides for NBD, which is an environment-sensitive probe that has been previously used in protein ligand-receptor studies (29). The fluorescence recovery achieved for titration of carboxylate groups was about 40% higher than the titration of amino groups in the case of ATS slides. A 2-fold increase would be expected, assuming that each surface amino group reacts with one molecule of BTA to leave two accessible carboxylate groups. The lower yield observed could be a result of the attachment of

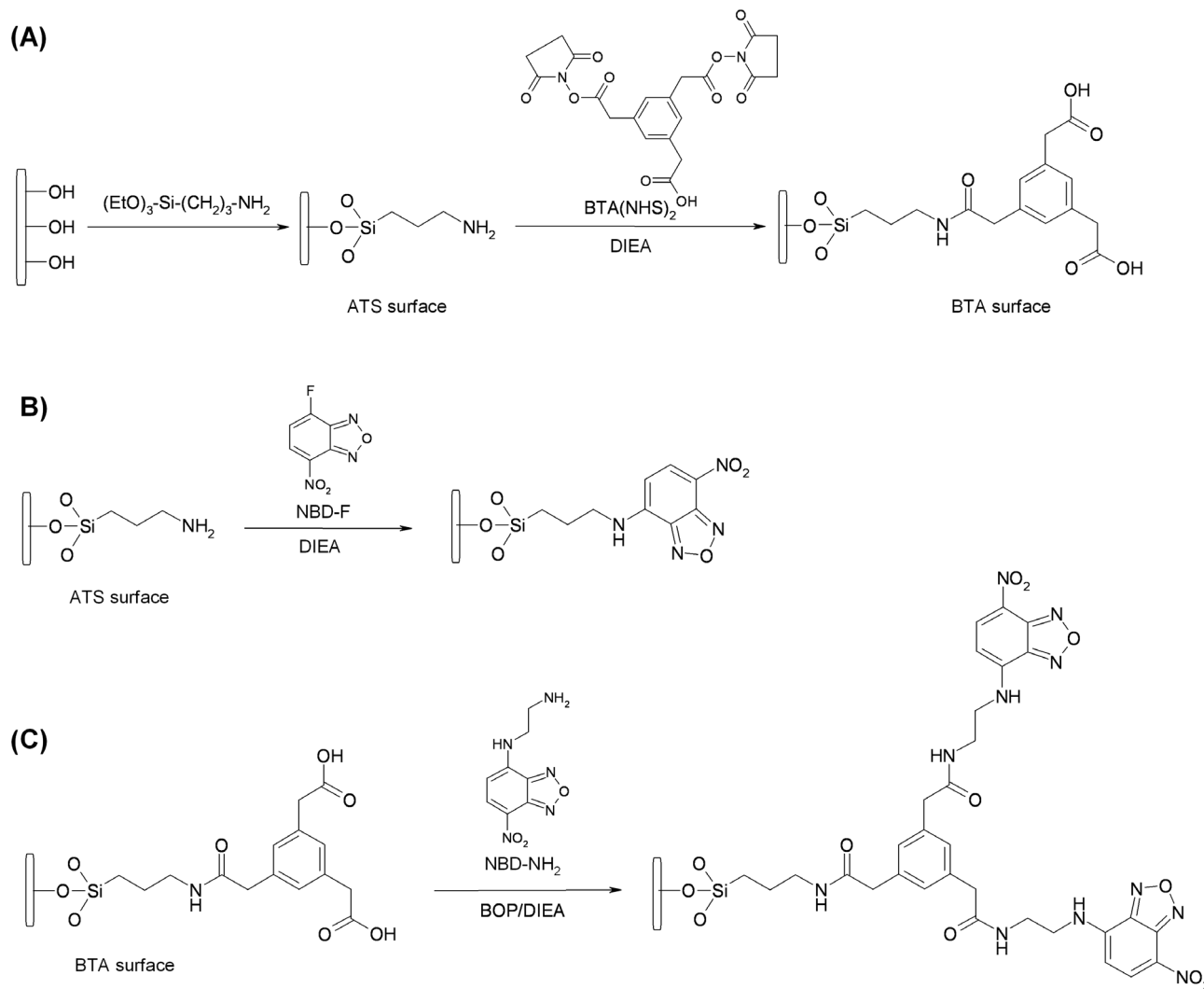


Figure 1. (A) Simplified diagram of steps required for glass functionalization with amino (ATS) and carboxyl (BTA) groups. Staining of derivatized glass surfaces using NBD reagents of (B) aminated (ATS) and (C) carboxylated (BTA) glass slides.

some BTA molecules through two carboxylate groups, and, to a minor extent, a result of unreacted amino groups.

We have also made an attempt to quantify the amount of amino and carboxyl groups on glass surfaces using the commercially available activated dye, Texas Red[®]-X succinimidyl ester (sc-TR), in addition to the aminated Texas Red dye in the presence of BOP/DIEA or DCC/DIEA catalysts. No dependence was found between the fluorescence intensity of grafted Texas Red amine, and the amount of surface carboxyl groups, for derivatization times that ranged from 5 min to 1 h. Furthermore, sc-TR-staining of ATS slides gave only slightly greater signal than the negative control on carboxylated BTA glass slides (data not shown). This is likely a result of strong electrostatic interactions between the ionized Texas Red dye and charged BTA and ATS surfaces. Indeed, this renders washing stained slides very difficult and in order to remove the dye from the surface, stained slides had to be shaken in a NaCl solution (1 M) for more than 12 h. This led to almost complete disappearance of fluorescence, which would suggest that the dye was adsorbed rather than covalently attached to glass surface. Because of the above-mentioned problems,

we have discontinued the use of Texas Red and have switched to non-charged NBD derivatives, which seem to be more appropriate probes for quantifying the efficiency of the amine-into-carboxyl-group conversion on glass surfaces.

Attachment of oligonucleotides to the BTA-functionalized glass surface

The hybridization of fluorescently modified DNA probes to surface-attached oligonucleotides was used extensively in the present work for further characterization and optimization of BTA carboxylation and DNA attachment conditions. Control experiments for DNA attachment on BTA surface showed that no signals above background levels were obtained if the reaction of an aminated oligonucleotide is performed in the absence of the coupling agent (EDC/1-methyl-imidazole). Experiments using, underivatized 5'-OH or 5'-SH derivatized oligonucleotides in the presence of EDC/1-methyl-imidazole coupling agent did not yield any significant signal upon hybridization with a fluorescently labeled complementary probe. This suggests that the material is mainly attached

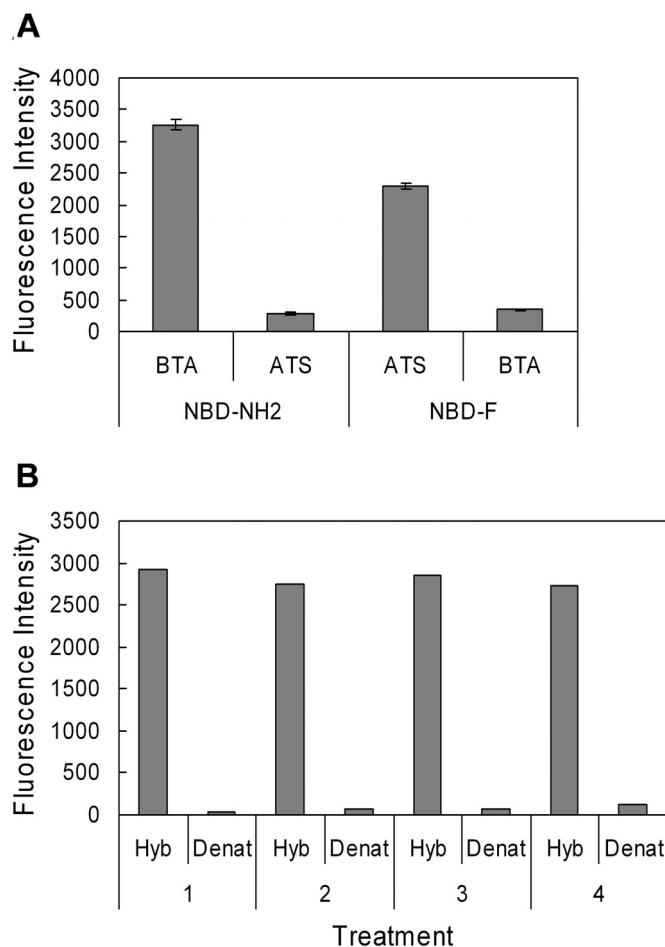


Figure 2. Characterization of BTA-derivatized slides (A) Slide staining with NBD. Example of staining of aminosilanized (ATS) slides with (A) 4-fluoro-7-nitrobenzofurazan (NBD-F) and of BTA slides with (7-nitrobenzo-2-oxa-1,3-diazol-4-yl)ethylenediamine (NBD-NH₂), and corresponding negative controls. Fluorescence of NBD-modified slides was measured in air with 5 s acquisition time, using the optical filter xf22 from Omega. (B) Consecutive hybridizations using a complementary TR-labeled probe. Four successive treatments of hybridization-denaturation (1 to 4) of the Texas Red-labeled reverse-P1 primer (500 nM) shown for 5'-amino-10T-P1 primer (34mer) grafted at 1 μ M for 60 min. Hybridization conditions: 500 nM Texas Red-labeled oligonucleotide in TMN buffer. Denaturation conditions: three times soaking in 50% formamide (v/v in H₂O) for 5 min at 80°C.

through a 5'-amino DNA ruling out non-specific reactions or interaction mechanisms.

Concentration dependence for grafting of the 5'-amino-10T-P2 primer (34mer) together with time dependence for grafting of the 5'-amino-10T-SS-P2 primer on BTA glass are shown in Figure 3. The plateau for the maximum surface coverage in the case of grafting the 5'-amino-10T-P2 primer was attained at a primer concentration in solution of 1 μ M (Figure 3A). Grafting kinetics of 5'-amino-10T-SS-P2 (Figure 3B) as well as that of 5'-amino-10T-P2 (data not shown) were found to be similar on BTA glass and polymeric Nucleolink wells (NUNC) with primer at 1 μ M concentration, and grafting conditions of EDC (10 mM)1-methyl-imidazole (10 mM) at 50°C as described previously (12). Specifically, the saturation coverage under the latter conditions was possible for grafting times of about 1 h.

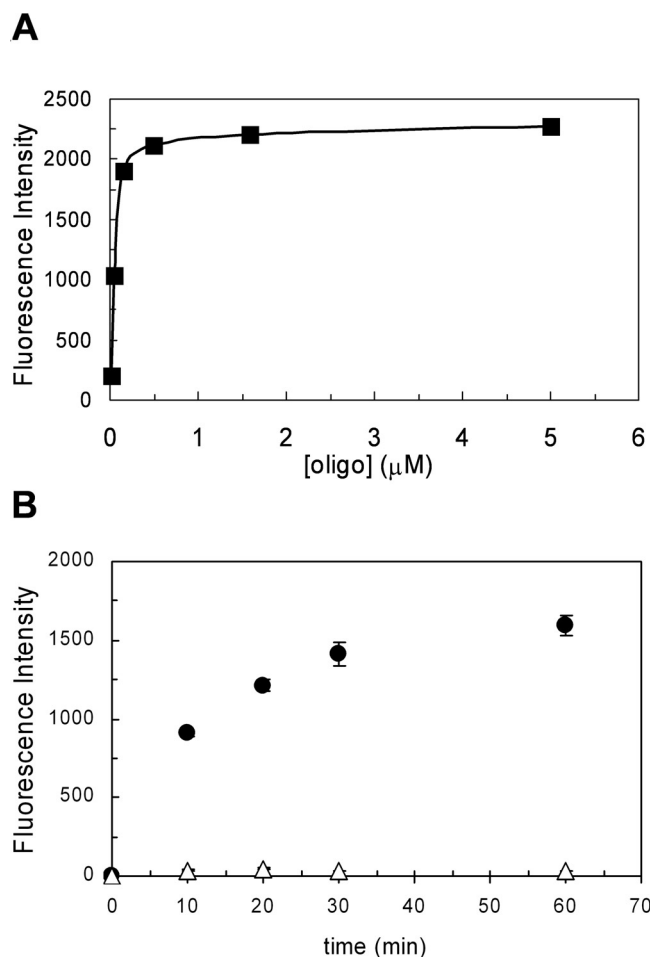


Figure 3. Probe concentration and time dependence for the attachment of aminated oligonucleotides to BTA-derivatized slides monitored by hybridization using a TR-labeled oligonucleotide. (A) Concentration dependence for the grafting of 5'-amino-10T-P₂ primer (34mer) on BTA glass. Grafting conditions: 10 mM EDC/10 mM 1-Methyl-imidazole (50°C)/1 h. Hybridization: 500 nM reverse-P₂-Texas Red in TMN buffer. The solid line is to guide the eye. Background fluorescence is subtracted. (B) Time dependence for the grafting of 5'-amino-10T-SS-P₂ primer on BTA glass. Grafting conditions: 1 μ M primer, 10 mM EDC/10 mM 1-Methyl-Imidazole (50°C at different incubation times. Hybridization (circles), 500 nM reverse-P₂-Texas Red in TMN buffer. Denaturation (triangle): three times, 5 min at 80°C in 50% formamide (v/v in H₂O). Experimental data points are background subtracted.

Analytical methods for DNA chip content characterization

In order to analyze the DNA templates attached on the surface before and after amplification we developed two different methods. In a first approach, we applied asymmetric PCR using one amplification probe labeled with Cy 5.0 that generate multiple copies in solution of the attached DNA sequence for subsequent gel electrophoresis analysis. The size of the recovered material depends on the sequence of the probe that will hybridize the attached templates at selected positions. The full size template is obtained for 3' specific oligonucleotide probes. This content amplification method has been used as an amplification QC method and proved to be a useful tool for further optimization of the amplification process. However, the method is not informative about possible contaminants lacking the sequence to be primed by the oligonucleotide

used for the asymmetric PCR. This fact prompted us to develop a more direct assay for the surface-amplified DNA. In this second QC analysis, the entire DNA attached on the surface using a disulfide linker is labeled at its 3'-OH position with ddNTP-Cy 5.0 or [α - 33 P]ddNTPs using terminal deoxynucleotidyl transferase (TdT labeling) and the labeled material released after reductive treatment is further analyzed by gel electrophoresis.

Another advantage of this direct-labeling approach over PCR-based QC methods is that no artifacts are introduced during the QC from the thermally surface-released DNA and its eventual extension or amplification in the solution phase. TdT labeling can be quite useful and informative for the determination of ratios of grafted template and primers provided, both contain groups that are cleavable under mild conditions, such as disulfides, vicinal diols, RNA linkers or protease motifs. As this method is 10-fold less sensitive than the asymmetric PCR approach (10-fold amplification expected using 10 cycles of linear amplification), it may be used to perform QC on cleavable DNA colonies (Figure 8) or in cases where the surface concentration of DNA template is relatively high. While we were unable to detect a disulfide linked template (462mer), or disulfide 80mer synthetic oligonucleotide (T2) using TdT labeling approach (both having been grafted onto BTA surfaces from 1.25 nM solutions, it was possible to detect the latter template when grafted onto BTA from a higher solution concentration (130 nM) (Figure 4). It is important to note that the TdT-mediated labeling reaction at 37°C takes place at a relatively low temperature, at which practically no thermally induced release of grafted template from the glass surface has been observed (in contrast to PCR thermocycling, which requires temperatures between 60 and 95°C).

BTA chemistry applied to microfabricated channels

Glass channels were validated using the same QC criteria that were applied for glass slides, and showed identical grafting density and specificity of attached oligonucleotides as

well as comparable colony density. The reproducibility of glass functionalization and oligonucleotide attachment was assessed by the hybridization of a fluorescent probe complementary to the 5'-amine-terminated oligonucleotide attached to the carboxyl groups after BTA reaction. Two criteria were considered in the determination of experimental variability; total fluorescence recovered after irradiation, and homogeneity of the image. Within an image (0.143 mm²) the standard deviation/mean fluorescence was less than 3%. From images taken at four different positions along a channel for eight channels (32 images), the standard deviation obtained was less than 4% of the averaged mean fluorescence values. The same result was obtained for chip-to-chip variability as determined by three independent experiments.

Stability tests were conducted with BTA-functionalized glass channels stored over various periods of time under dry vacuum. Similar results were obtained for all the time points evaluated, as determined by the readout from the hybridization of a fluorescent probe to a freshly grafted 5'-aminated oligonucleotide. The values obtained were within the range of channel-to-channel reproducibility for freshly BTA-functionalized surfaces. From these stability tests we concluded that BTA-derivatized glass channels could be stored under vacuum for at least 8 months.

Density of attachment and thermal stability of BTA chemistry

The surface density of amplification primers is of paramount importance for the template bridging process during solid-phase amplification. The surface coverage of BTA-grafted oligonucleotides was compared with other carboxylate linker chemistries using glass slides. Identical oligonucleotide attachment conditions were applied to BTA-derivatized slides; aminosilanized slides treated with phenyleneisothiocyanate (PCITC) and commercially available carboxyl-terminated slides CAB-25C from CEL Associates. The fluorescence signals recover for BTA-treated slides was at least 2-fold

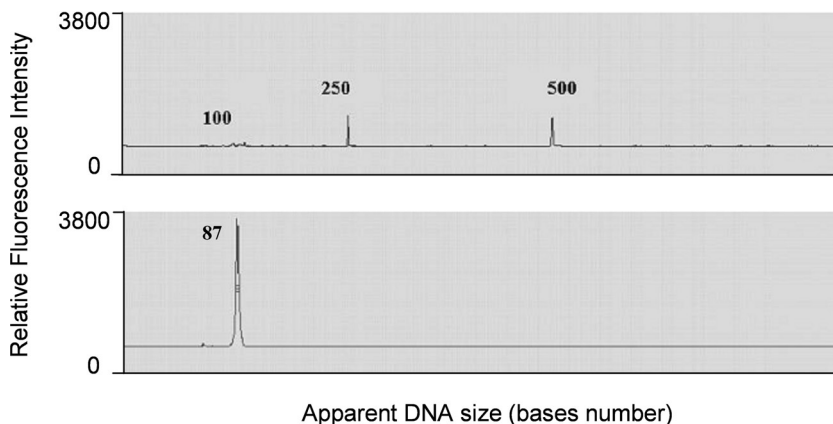


Figure 4. Surface DNA content analysis by TdT labeling of 3'-OH using Cy5-ddNTP Visual Genetics sequencer trace recorded for disulfide-T2 template (80mer) grafted on BTA glass, labeled with 500 nM ddNTP-Cy5.0 at 3' end (TdT) and cleaved from the surface in 50 mM DTT/Tris solution pH 8.5 (1 h). Grafting conditions: 130 nM 5'-amino-SS-T2 template, 10 mM EDC/10 mM 1-Methyl-Imidazole (50°C)/1 h. Terminal Deoxynucleotidyl Transferase (TdT), 20 μ /ml, NEB buffer4, CoCl₂ (250 mM). Sequencer (Visible Genetics, VG90008), SureFill 6% Sequencing Gel (Visible Genetics, Ref. #VG40006), Stop Loading Dye (Amersham). The upper traces represent DNA size markers labeled with Cy5.5. In the lower trace, the Cy5 labelled oligonucleotide extracted from the surface migrates as a single peak with an approximate apparent size of 87 bases.

higher than PCITC slides, and about 10-fold higher than commercial slides.

BTA coatings were compared with other chemistries developed in house, with regards to DNA surface attachment densities in glass channels, and thermal stability when subjected to a typical thermocycling protocol for solid-phase amplification of DNA (Figure 5). In the three cases studied, the ATS-derivatized glass underwent further treatment with the cross-linking reagents BTA (Figure 1), TMA or s-MBS (12). Single-stranded primer (P1) was then attached according to the specific chemistry, 5'-NH₂ for BTA and TMA, or 5'-SH for s-MBS. The grafting density was then assessed by hybridization with a complementary oligonucleotide (revP1) labeled with Texas Red[®]-X at its 5' position, before and after the thermal treatment. The samples were thermocycled with the following cycle: 45 s, 97.5°C; 90 s, 60°C; 90 s, 73°C over 40 iterations.

TMA and BTA showed comparable densities of attachment that were 8- and 10-fold higher, respectively, than the coverage obtained using the heterobifunctional cross-linking reagent s-MBS, as illustrated in Figure 5. The loss of grafted primer from thermal cycling was nearly 60%, independent of the linker tested. Our results would suggest that the release of attached DNA may take place at the silane level supported by previously reported data showing that silane layers are subject to hydrolysis upon thermal treatments (30). It has been reported for similar thermal stability experiments applied to s-MBS chemistry that the most significant release of DNA occurs after the first cycle, which reveals the instability of the surface conditioning under elevated temperatures (27).

The thermal lability of attached DNA molecules could have two effects in the DNA amplification process: a reduction in the amplification yield, and the generation of contaminants from the released DNA material. The latter effect is potentially more dangerous. Toward elimination of the contamination risk posed by released DNA, we took advantage of the integrated fluidic system to flush the channels during the most critical part of the process; the denaturing step. In addition, an empirical selection of designed thermal cycles and iteration numbers allowed us to optimize the amplification conditions for subsequent detection by chip content analytical procedures,

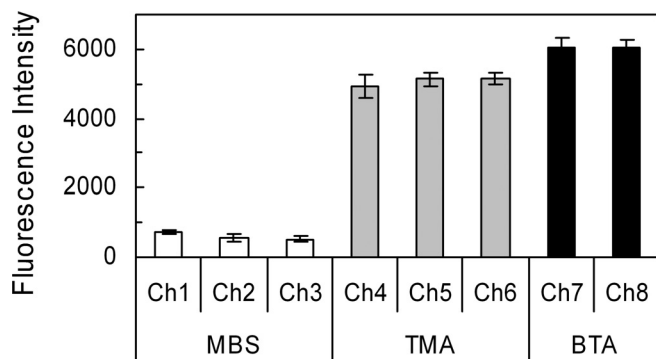


Figure 5. Yield of DNA attachment using different cross-linkers between aminated slides (ATS) and modified oligonucleotides. Experiment is performed in one glass channel chip. The channels differ by the cross-linker used: First 3 channels are treated with s-MBS. Channels 4 to 6 are treated with TMA and 7–8 with BTA. The surfaces are then grafted and hybridized with the appropriate DNA material (see text for grafting and hybridization conditions).

fluorescent hybridization and fluorescent sequencing by synthesis of surface-amplified DNA (to be reported elsewhere). The thermal stability of the attached DNA primers has also been tested for successive treatments of hybridization and denaturation (Figure 2B). The recovery of fluorescence slightly decreased after a total incubation time of 20 min at 80°C during the four consecutive treatments, indicating a good thermal stability towards the denaturing conditions employed. These results demonstrate that recycling the DNA-derivatized glass using BTA chemistry is possible for experiments, such as hybridization-based assays.

Solid-phase amplification of DNA on BTA glass and formation of DNA colonies

Colonies were generated with BTA-treated glass channels that were subsequently co-grafted with aminated amplification primers and aminated template. Thermocycling was carried out with *Taq* polymerase. The primer/template concentration ratio in solution defined the number of initially attached single-template molecules, and therefore the DNA colony surface density after amplification. Experiments at different primer/template concentration ratios and different cycle numbers were conducted for the ultimate optimization of the process. Figure 6 shows images of colonies generated after 40 amplification cycles with grafting concentrations of 1 μM for amplification primers and 50 pM for the DNA template. DNA Colonies visualized after Sybr-Green I staining, a dsDNA specific dye (31), allowed to determine the colony density achieved for these experimental conditions that was about 10 million colonies/cm². The trace A of the histogram in Figure 6 represents the colony intensity distribution for the whole image in panel (A).

In situ enzymatic digestion of surface-amplified dsDNA colonies using BbvI

After solid-phase amplification, dsDNA colonies generated can be cleaved by restriction enzymes at selected positions of the template sequence. The analysis of fragmented DNA provides another useful analytical method for determination of the quality of the DNA copies after the *in situ* amplification process. Indeed, selection of restriction enzymes for given positions in the original DNA template sequence, affords the ability to check the size of expected DNA fragments after digestion, with the QC methods for content analysis discussed above (TdT labeling or asymmetric PCR). Furthermore, the analytical tools developed allowed optimization of the experimental conditions, such as enzyme concentration, buffer composition and reaction time, for efficient restriction enzyme cleavage.

The digestion of dsDNA arrays with DpnI has been reported for studying interactions of processed DNA with DNA-binding proteins (32). In our lab, DNA colonies have been subjected to digestion assays with several type-I restriction enzymes as well as the type IIs-restriction enzymes BbvI. Herein we focus on the BbvI results, as this enzyme, in addition to other type IIs enzymes (e.g. FokI) can be used in massively parallel sequencing (MPSS) technique (7) that was investigated in our lab for its implementation in conjunction with DNA colonies. BbvI creates dsDNA breaks with a 4-base 3' overhang. The recognition sequence is separated

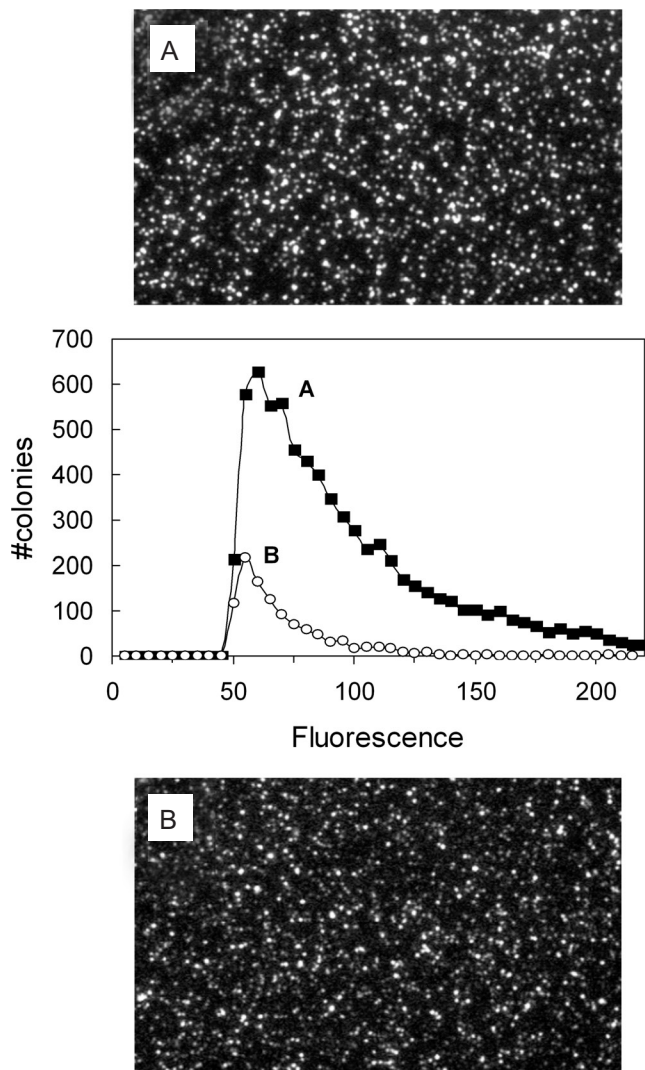


Figure 6. Characterization of DNA colonies generated using BTA chemistry by Sybr-Green I staining. Histograms of colonies average intensity (A) Before BbvI digestion colonies are double stranded and their length is 347 bp. (B) After BbvI digestion, colonies are still double stranded and their length is reduced to 43 bp, showing consequently a lower average intensity.

from the cleavage site by 8 bases on one strand, and 12 on the other. Colonies were created with a DNA template containing a unique BbvI recognition site (Figure 7A) and amplification primers, wherein one was surface-bound through a cleavable disulfide bond. This permitted the selective removal of one of the cleaved fragments from the surface after reductive (non-denaturing) treatment. BbvI cleaves the full size (347 bp) template at one site to give products of 43 and 300 bp, respectively (plus 4-base 3'-overhangs). The larger fragment attached to the surface by disulfide bonds is removed after DTT treatment, whereas the shorter dsDNA remains attached to the surface, and can be detected by Sybr-Green I stain (Figure 6B and its corresponding histogram trace). The histogram B corresponds to a distribution of colony intensities that are weaker than those in the distribution for full-length colonies (Figure 6A). The Sybr-Green I fluorescence intensity is proportional to the concentration of dsDNA and the number of base pairs. In the experimental example of Figure 6, the

surface concentration of DNA remained practically unchanged, as under the experimental conditions of the assay, there is no DNA released from the surface. Therefore, as expected, the histogram evolution reflects the reduction of template size of about 85% for the processed colonies.

Undigested colonies, or colonies having been exposed to varying BbvI concentrations, were 3' end-labeled with [α - 32 P]ddNTPs using the TdT labeling method. Product DNA was simultaneously cleaved from the surface, and denatured from complementary sequences by introducing a solution of DTT in formamide-containing gel-loading buffer (Figure 7). The glass channel surface was heated to 95°C, and the labeled DNA products collected for loading onto a 10% polyacrylamide denaturing gel. The resulting autoradiogram is shown in Figure 8A, and represents the products of various BbvI concentrations for reactions carried out at 37°C for 1 h. Under these conditions, a BbvI concentration of 0.25 U/ μ l generated nearly quantitative cleavage of colony DNA as illustrated in the autoradiogram by the band shift after enzymatic digestion. Radioactive gel quantitation gave product /substrate ratios that correlated well with those determined by fluorescence-based (Cy 5.0) 3' end labeling of products from similar reactions. For TdT labeling with Cy 5.0, a similar procedure has been used, except that the released labeled material was analyzed using a Visible Genetics sequencer equipped with a two-channel detection unit corresponding to Cy 5.0 and Cy 5.5; the latter dye was used as a size marker in the electropherograms (Figure 8B). Both readout techniques indicate that total conversion to cleaved product can occur given sufficient time, and using relatively small amounts of BbvI. More important than the reaction time or the amount of enzyme required to yield full cleavage of DNA, is the fact that within our limits of detection, all DNA strands in the colonies are viable substrates for enzyme processing. This type of uniformity and consistency are important to the success of the DNA colonies in their application in massively parallel sequencing efforts, such as the implementation of MPSS sequencing or the application of *in situ* sequencing by synthesis methods.

CONCLUSIONS

We found a new chemical cross-linker for 5'-aminated DNA immobilization on amino silanized glass surfaces that fulfill the requirements of solid-phase PCR for the generation of DNA colonies. The BTA cross-linker provides robust and reproducible derivatization chemistry and we demonstrated that the multi step process of DNA surface derivatization can be miniaturized and automated for its utilization in high-throughput DNA analysis thanks to the use of fluidic integrated microfabricated glass channels.

Density of attached amplification primers that is of great importance for an efficient amplification process, achieved with BTA provides the most adequate levels of surface functionalization on flat glass surfaces when compared with other cross-linkers (12,22). The DNA colony density achieved can be modulated by tuning the relative concentrations of primer/template initially attached, in particular for a given optimal concentration of amplification primers (1 μ M in solution), the variation of template concentration affects the initial surface

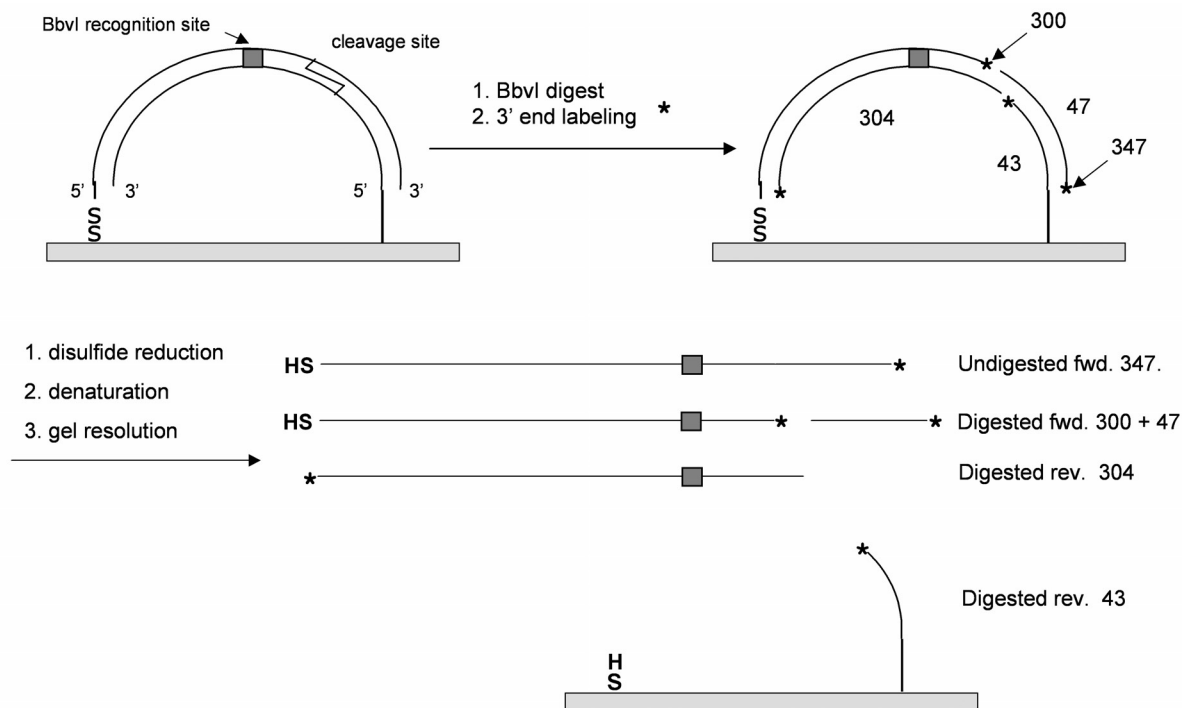


Figure 7. *In situ* restriction digestion of dsDNA colonies. Analysis of products resulting from *in situ* enzymatic digestions of solid-phase-amplified dsDNA. Numbers on the right indicate approximate base lengths of BbvI cleavage products harvested from the surface.

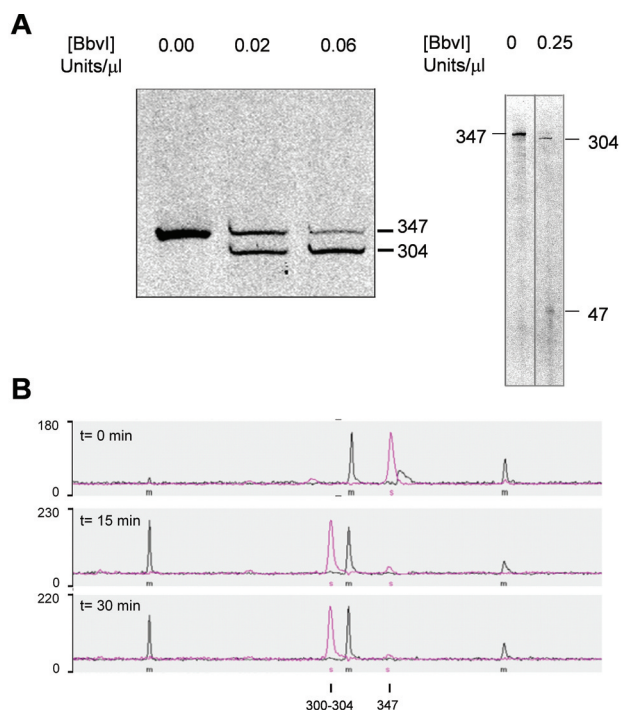


Figure 8. Characterization of *in situ* restriction digestion of dsDNA colonies. (A) Autoradiograms obtained from surface-released products labeled at their 3' ends with [α - 32 P]ddNTP. Digestions were performed for 1 h at various concentrations (indicated) of BbvI. The gel images show DNA cleavage as a function of enzyme concentration (left) and near-quantitative cleavage of the 347-bases substrate to the expected products of 300–304 and 47 bases (right). (B) Electropherograms obtained from surface-released products at different enzymatic reaction times for 0.25 μ l BbvI followed by 3' end labeling with ddNTP-Cy5.

density of single-molecules of templates to be amplified, therefore the DNA colonies final density. We have optimized the experimental conditions for reaching final DNA colonies densities of about 1×10^7 colonies/cm² which represents the highest density ever reported for *in situ* DNA amplification. Higher levels of surface density are possible using BTA chemistry but not recommended because of image analysis issues related to the formation of adjacent colonies.

Parameters such as the integrity of amplified material, good specificity of 5' attachment of DNA and extent of amplification can be evaluated by the application of the methods we developed for analyzing surface-amplified DNA. Moreover, these methods and in particular the direct TdT labeling approach were utilized during the optimization process and were used to analyze the purity of the amplified material from initial single DNA template molecules attached and for the analysis of *in situ* digested dsDNA colonies. To our knowledge, this is the first report where 'total DNA chip content analytical procedures' have been applied for analyzing surface immobilized DNA.

The thermal lability of the surface is mainly due to silane layer therefore it could not be improved by the exchange of the cross-linker moiety between the silane and the DNA to be attached. This suggests that further improvements in the thermal stability of attached material should come from the application of different chip substrates such as functionalized polymers. Moreover, for process industrialization reasons, the use of a cheap disposable material would be beneficial.

It has been shown in the present work that the DNA colonies are amenable to quantitative enzymatic *in situ* digestion and eventually to high-throughput sequencing by applying the MPSS method (7). The MPSS method consists in the

application of repetitive steps of type IIs enzymatic digestions and ligation of encoded adapters. Preliminary results obtained in our lab for the integration of MPSS to DNA colonies showed the compatibility of both approaches and their potential further development for large-scale genomic analysis applications.

The application of sequencing by synthesis methods using fluorescent nucleotides acting as transient terminators has also been explored in our lab for obtaining a binary signal detection method with various applications in the genomic field.

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Conflict of interest statement. Manteia, a Swiss-based private company developing an ultra high-throughput DNA sequencing technology has been acquired jointly by Lynx Therapeutics, Inc. www.lynxgen.com and Solexa Ltd www.solexa.com. Both companies completed a merger in March 2005 and became Solexa Inc.

REFERENCES

- Shendure, J., Mitra, R.D., Varma, C. and Church, G.M. (2004) Advanced sequencing technologies: Methods and goals. *Nature Rev. Genet.*, **5**, 335–344.
- Carlson, C.S., Eberle, M.A., Kruglyak, L. and Nickerson, D.A. (2004) Mapping complex disease loci in whole-genome association studies. *Nature*, **429**, 446–452.
- Devlin, B., Roeder, K. and Wasserman, L. (2001) Genomic control, a new approach to genetic-based association studies. *Theor. Popul. Biol.*, **60**, 155–166.
- Dobrin, S.E. and Stephan, D.A. (2003) Integrating microarrays into disease-gene identification strategies. *Expert Rev. Mol. Diagn.*, **3**, 375–385.
- Tebbutt, S.J., He, J.Q., Burkett, K.M., Juan, J., Opushnyev, I.V., Tripp, B.W., Zeznik, J.A., Abara, C.O., Nelson, C.C. and Walley, K.R. (2004) Microarray genotyping resource to determine population stratification in genetic association studies of complex disease. *Biotechniques*, **37**, 977–985.
- Zeng, D. and Lin, D.Y. (2005) Estimating haplotype-disease associations with pooled genotype data. *Genet. Epidemiol.*, **28**, 70–82.
- Brenner, S., Johnson, M., Bridgham, J., Golda, G., Lloyd, D.H., Johnson, D., Luo, S.J., McCurdy, S., Foy, M., Ewan, M. *et al.* (2000) Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nat. Biotechnol.*, **18**, 630–634.
- Marziali, A. and Akeson, M. (2001) New DNA sequencing methods. *Ann. Rev. Biomed. Eng.*, **3**, 195–223.
- Meyers, B.C., Tej, S.S., Vu, T.H., Haudenschild, C.D., Agrawal, V., Edberg, S.B., Ghazal, H. and Decola, S. (2004) The use of MPSS for whole-genome transcriptional analysis in *Arabidopsis*. *Genome Res.*, **14**, 1641–1653.
- Silva, A.P.M., Chen, J.J., Carraro, D.M., Wang, S.M. and Camargo, A.A. (2004) Generation of longer 3' cDNA fragments from massively parallel signature sequencing tags. *Nucleic Acids Res.*, **32**, e94.
- Silva, A.P.M., De Souza, J.E.S., Galante, P.A.F., Riggins, G.J., De Souza, S.J. and Camargo, A.A. (2004) The impact of SNPs on the interpretation of SAGE and MPSS experimental data. *Nucleic Acids Res.*, **32**, 6104–6110.
- Adessi, C., Matton, G., Ayala, G., Turcatti, G., Mermod, J.-J., Mayer, P. and Kawashima, E. (2000) Solid-phase DNA amplification: characterisation of primer attachment and amplification mechanisms. *Nucleic Acids Res.*, **28**, E87.
- Mitra, R.D., Shendure, J., Olejnik, J., Edyta Krzymanska, O. and Church, G.M. (2003) Fluorescent *in situ* sequencing on polymerase colonies. *Anal. Biochem.*, **320**, 55–65.
- Shendure, J., Porreca, G.J., Reppas, N.B., Lin, X., McCutcheon, J.P., Rosenbaum, A.M., Wang, M.D., Zhang, K., Mitra, R.D. and Church, G.M. (2005) Accurate multiplex polony sequencing of an evolved bacterial genome. *Science*, **309**, 1728–1732.
- Steinberg, G., Steinberg, K., Thomas, L., Barker, D. and Zhao, C. (2004) Strategies for covalent attachment of DNA to beads. *Biopolymers*, **73**, 597–605.
- Beaucage, S.L. (2001) Strategies in the preparation of DNA oligonucleotide arrays for diagnostic applications. *Curr. Med. Chem.*, **8**, 1213–1244.
- Defrancq, E., Hoang, A., Vinet, F. and Dumy, P. (2003) Oxime bond formation for the covalent attachment of oligonucleotides on glass support. *Bioorg. Med. Chem. Lett.*, **13**, 2683.
- Lee, P.H., Sawan, S.P., Modrusan, Z., Arnold, L.J., Jr and Reynolds, M.A. (2002) An efficient binding chemistry for glass polynucleotide microarrays. *Bioconjug. Chem.*, **13**, 97–103.
- Zammatteo, N., Jeanmart, L., Hamels, S., Courtois, S., Louette, P., Hevesi, L. and Remacle, J. (2000) Comparison between different strategies of covalent attachment of DNA to glass surfaces to build DNA microarrays. *Anal. Biochem.*, **280**, 143.
- Andreadis, J.D. and Chrisey, L.A. (2000) Use of immobilized PCR primers to generate covalently immobilized DNAs for *in vitro* transcription/translation reactions. *Nucleic Acids Res.*, **28**, e5.
- Smith, L., Underhill, P., Pritchard, C., Tymowska-Lalanne, Z., Abdul-Hussein, S., Hilton, H., Winchester, L., Williams, D., Freeman, T., Webb, S. *et al.* (2003) Single primer amplification (SPA) of cDNA for microarray expression analysis. *Nucleic Acids Res.*, **31**, e9.
- Lindroos, K., Liljedahl, U., Raitio, M. and Syvanen, A.-C. (2001) Minisequencing on oligonucleotide microarrays: comparison of immobilisation chemistries. *Nucleic Acids Res.*, **29**, e69.
- Lefevre, C., Kang, H.C., Haugland, R.P., Malekzadeh, N., Arttamangkul, S. and Haugland, R.P. (1996) Texas red-X and rhodamine red-X, new derivatives of sulforhodamine 101 and lissamine rhodamine B with improved labeling and fluorescence properties. *Bioconjug. Chem.*, **7**, 482–489.
- Mujumdar, R.B., Ernst, L.A., Mujumdar, S.R., Lewis, C.J. and Waggoner, A.S. (1993) Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters. *Bioconjug. Chem.*, **4**, 105–111.
- Duthie, R.S., Kalve, I.M., Samols, S.B., Hamilton, S., Livshin, I., Khot, M., Nampalli, S., Kumar, S. and Fuller, C.W. (2002) Novel cyanine dye-labeled dideoxynucleoside triphosphates for DNA sequencing. *Bioconjug. Chem.*, **13**, 699–706.
- Kovacs, G.T.A. (1998) *Micromachined Transducers Sourcebook*. WCB McGraw-Hill, Boston, MA.
- Dodge, A., Turcatti, G., Lawrence, I., de Rooij, N.F. and Verpoorte, E. (2004) A microfluidic platform using molecular beacon-based temperature calibration for thermal dehybridization of surface-bound DNA. *Anal. Chem.*, **76**, 1778–1787.
- Han, S.-Y. and Kim, Y.-A. (2004) Recent development of peptide coupling reagents in organic synthesis. *Tetrahedron*, **60**, 24447–22467.
- Turcatti, G., Vogel, H. and Chollet, A. (1995) Probing the binding domain of the Nk2 receptor with fluorescent ligands: evidence that heptapeptide agonists and antagonists bind differently. *Biochemistry*, **34**, 3972–3980.
- Yim, H., Kent, M.S., Tallant, D.R., Garcia, M.J. and Majewski, J. (2005) Hygrothermal degradation of (3-Glycidoxypoly)trimethoxysilane films studied by neutron and X-ray reflectivity and attenuated total reflection infrared spectroscopy. *Langmuir*, **21**, 4382–4392.
- Zipper, H., Brunner, H., Bernhagen, J. and Vitzthum, F. (2004) Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Res.*, **32**, e103.
- Bulyk, M.L., Gentalen, E., Lockhart, D.J. and Church, G.M. (1999) Quantifying DNA-protein interactions by double-stranded DNA arrays. *Nat. Biotechnol.*, **17**, 573–577.