Chapter 3

The "Clickable" Method for Oligonucleotide Immobilization Onto Azide-Functionalized Microarrays

Tomasz Ratajczak*, Barbara Uszczyńska*, Emilia Frydrych-Tomczak, and Marcin K. Chmielewski

Abstract

The DNA microarray technique was supposed to help identifying and analyzing the expression level of tens of thousands of genes in the whole genome. But there is a serious problem concerning fabrication of the microarrays by chemical synthesis, such as specific and efficient linking of probes to a solid support. Therefore, we reckon that applying "click" chemistry to covalently anchor oligonucleotides on chemically modified supports may help construct microarrays in applications such as gene identification. Silanization of the glass support with organofunctional silane makes it possible to link azide groups on glass surface and the nucleic acid probe that is equipped with a pentynyl group. This is followed by direct spotting of the nucleic acid on the azide-modified glass support in the presence of copper ions, and this is a frequently applied method of "click" chemistry.

Key words Click chemistry, Oligonucleotides, DNA, Microarrays, Silanization, Hybridization, Fluorescence, Phosphoramidite

1 Introduction

A microarray is defined as a collection of probes (nucleic acids, proteins, lipids, peptides, or carbohydrates) immobilized in an ordered 2-D manner on a solid support, e.g., plates, microchannels, microwells, or particles [1–4]. However, it is the nucleic acid microarrays on glass slides that are first developed and have found major application in biomedical research. The nucleic acid arrays enable assessment of gene expression, analysis of the genome structure, identification of genetic polymorphism, or detection of viral, bacterial, and fungal pathogens. The arrays give an opportunity to analyze thousands of different DNAs or RNAs in parallel with only a small amount of biological material [5]. In practice, there are two

*Author contributed equally with all other contributors.

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technologies for microarray fabrication: by synthesizing oligonucleotides "step by step" in situ or by spotting the previously synthesized oligonucleotides onto the surface of a slide [6, 7]. Currently, in situ synthesis method seems to be the most effective method for preparing high-density microarrays. This method uses a photolabile system of protection/deprotection hydroxyl groups. However, an alternative method for applying thermolabile protecting groups in the "heat-driven" approach has also been reported [8]. Spotting techniques are less effective but simpler, and consequently they are commonly used for the production of customized microarrays [9]. One of the key factors affecting the DNA microarray sensitivity and specificity is the method of probe immobilization on the solid support. Nucleic acid microarrays are being produced via electrostatic interactions or covalent bond formation between probes and solid support. The covalent binding of probes requires DNA to be equipped with a linker, which can form a stable covalent bond with groups located on a solid support [10]. Traditional spotting techniques use spotting pins that may not be small enough and this limitation restricts the size of microarrays. The atomic force microscope tips have been applied in the Dip-Pen Nanolithography (DPN) approach to the miniaturization of DNA nanoarrays [11]. However, the search for effective solutions in biopolymers immobilization on the solid phase is still a challenge in terms of performance and miniaturization of microarrays.

During the 2000s, Sharpless, Kolb, and Finn have introduced to the literature the concept of "click" chemistry [12]. It covers different condensation reactions that characterize rapidity, high efficiency, and specificity [13, 14]. To date, different variants of this "click" reaction are known (Fig. 1) [15], and it has also been

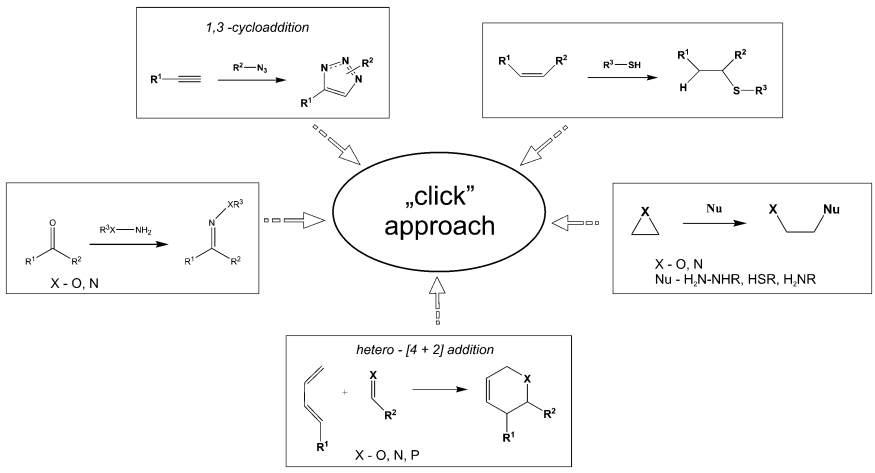

Fig. 1 Examples of reaction types that meet the descriptions of the "click" reaction

demonstrated that the "click" chemistry can be easily combined with microcontact printing [16]. Different types of cycloaddition in the "click" approach can be used for different applications.

The most suitable variant for microarray construction seems to be the copper (Cu(I))-catalyzed variant of Alkyne-Azide Cycloaddition (CuAAC) [17, 18], which was first reported for solid-phase synthesis of peptidotriazoles [19]. This method can be easily applied to attach alkyne or azide-modified oligonucleotides to well-defined surfaces with azide or terminal alkyne group, respectively [20]. The "click" reaction can occur in the presence of various agents, e.g., oxygen, water, and biological molecules [21]. For nucleic acid modifications with azide or alkynyl groups, there are two main strategies. The first one is based on using already modified phosphoramidite building blocks, which can be directly applied in automatic solid-phase DNA synthesis. This strategy provides good results and enables the synthesis of oligomers with an alkynyl group attached to 5′-OH of ribose [22], 2′-OH of ribose [23], the functional groups of nucleobases [24–27], or the internucleotide phosphate backbone [28]. Rozkiewicz et al. have immobilized 5′-alkynyl modified oligonucleotides on azide-bearing slides using microcontact printing. Synthesis in the nanoscale confinement between an elastomeric stamp and a reactive substrate leads to the formation of the desired product under ambient conditions, within a short time, and without a catalyst. The second strategy as applied by Ju et al. [29] is more complicated because the introduction of an azide group to the oligonucleotide during its chemical synthesis is inefficient due to the azide phosphoramidite instability [30]. However, the incorporation of the azide group can take place as a post-synthetic oligonucleotide modification, e.g., a bromine atom can be exchanged by the azide group [31].

We previously reported the application of the "click" reaction for the immobilization of DNA on a solid support [32]. Herein, we present a detailed protocol for this application. To this end, we chose the strategy of binding modified oligodeoxynucleotides (ODNs) (modified by an alkynyl group) to a solid surface covered with azide-functionalized silanes. Figure 2 shows schematically how the ODNs are linked with azide groups on the modified glass surface.

This method is simple and cheap because producing an azide-functionalized silane or a propargyl link is simple and the time required for effective functionalization of the support is short.

The phosphoramidite approach was used to introduce the alkynyl groups to oligodeoxynucleotides (ODN). Pentynyl phosphoramidite was first prepared and it was placed at the 5′-end of the ODN during the last step of chemical synthesis. Owing to the bifunctional nature of the azide-functionalized silane, i.e. 3-azidopropyltrimethoxysilane ($(CH_3O)_3Si(CH_2)_3N_3$) [33–35], it could be used as the "coupling agent" to link the biopolymeric moiety and inorganic silicon-based substrate (e.g., CPG particles or glass microscopic slides) [36].

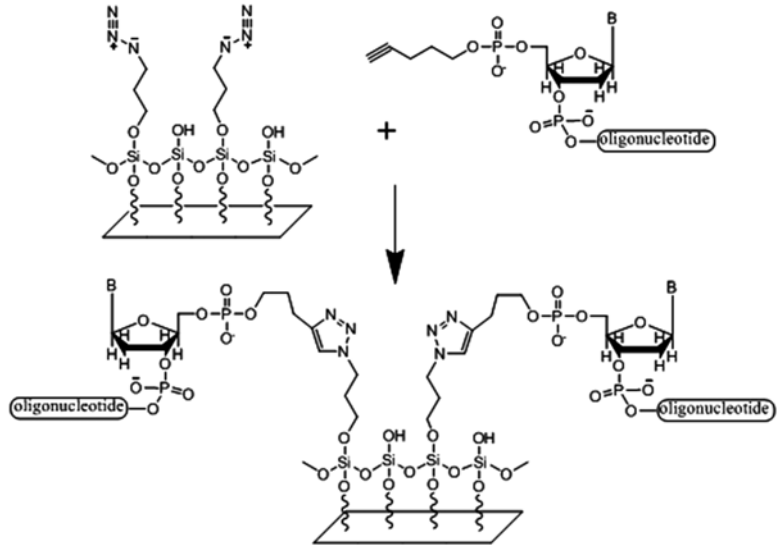

Fig. 2 Immobilization of a DNA oligodeoxynucleotide on the azide-functionalized glass surface (Reproduced from Ref. 32 with permission from Royal Society of Chemistry)

We spotted the modified ODN (with a pentynyl linker) on azide-functionalized slides in the presence of sodium ascorbate, $CuSO_4$, and glycerol. After intensive rinsing of the slide we carried out the hybridization procedure on printed slide with fluorescently labeled ODN targets. As shown in Fig. 3, 0.2 and 0.5 %, but not 0.02 %, of the silane gave hybridization signals that resulted from the formed duplexes.

2 Materials

2.1 Glass Substrate Preparation

1. Glass slides: standard soda-lime microscope slides (26 × 76 × 1 mm).
2. Ultrasonic cleaning units: Elma S60H, ultrasound frequency 37 kHz, ultrasonic power 150 W.
3. Acetone (analytical grade).
4. Microarray High-Throughput Wash Station (Arrayit Co., USA).
5. Magnetic stirrer.
6. Detergent solution: 4 % Trilux detergent in water. Add 250 mL of doubly distilled water to a high-throughput wash station, add 10 mL of Trilux detergent and mix.
7. Nitrogen gas (99.999 %) for drying.

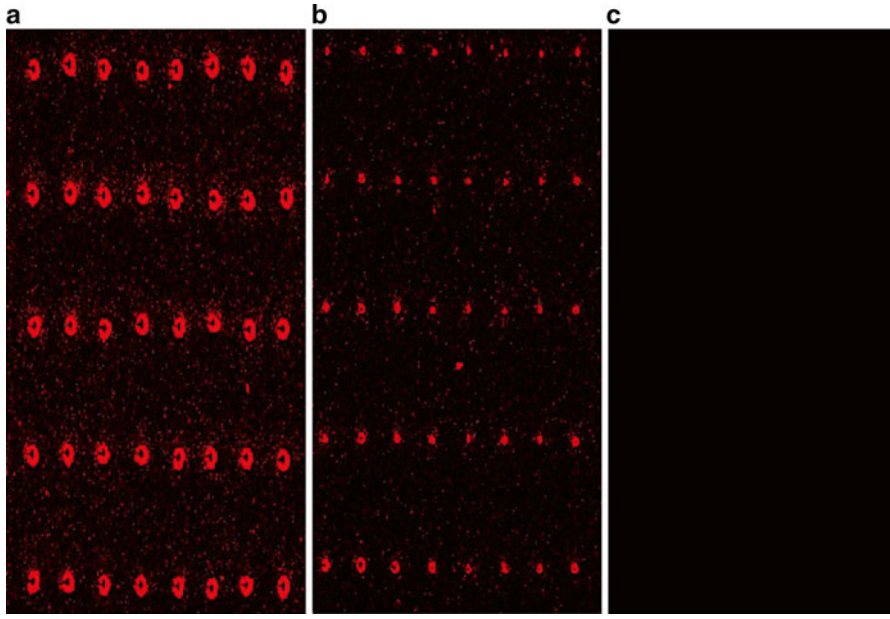

Fig. 3 Hybridization of fluorescently labeled targets with oligodeoxynucleotides that were immobilized via "click" chemistry on glass slides. Different concentrations of azide-functionalized silanes in toluene (v/v) (**a**) 0.5 % v/v, (**b**) 0.2 % v/v, (**c**) 0.02 % v/v were used. The targets are Cy5-labeled xxx. The distance between spots for each slide is 500 μm and the average SNR (signal-to-noise ratio) is 230 (Reproduced from Ref. 32 with permission from Royal Society of Chemistry)

2.2 Glass Substrate Silanization

1. Three Coplin staining jars each holding five slides.
2. Toluene pure.
3. Ethanol, absolut 99,8 % pure.
4. Silane solution for silanization of glass slides (0.02, 0.2, 0.5 % AzPTMS in toluene). Add 40 mL of toluene to three Coplin staining jars and put in 8×10^{-3}, 8×10^{-2}, 0.2 g AzPTMS, respectively, and mix.

2.3 CPG Silanization

1. Controlled pore glass (CPG): pore diameter (dp) = 125–160 μm;
   pore volume (Vp) = 1.06 cm^3 g^{-1}; particle diameter (D) = 94 nm (Cormay, Poland).
2. Round-bottom flask, 25 mL.
3. 3-azidopropyltrimethoxysilane (AzPTMS), synthesized according to the procedure developed by Mader et al. [37].
4. 5 % of AzPTMS in toluene, i.e., 0.25 g of AzPTMS was added to 5 mL of toluene in a round-bottom flask.

2.4 Synthesis of Modified Oligodeoxynucleotides (ODN)

1. 4-pentyl-1-ol, kept for 1 week over activated 3 Å sieves before use.
2. 3-hydroxypropionitrile, freshly distilled under reduced pressure and kept over activated 3 Å sieves before use.

3. Diisopropylamine (DIPEA), dried by distillation over NaH and kept over 3 Å activated sieves for 3 days before use.
4. Phosphorus trichloride (PCl_3) (*see* **Note 1**).
5. Benzene, dried by distillation over NaH and kept over 3 Å activated sieves for 5 days before use.
6. 10 mg of modified CPG solid supports (Biosearch Technologies Inc. USA) (*see* **Note 2**).
7. Oligodeoxynucleotides (ODNs), synthesized according to the procedure developed by *Caruthers* et al. [38] on a DNA synthesizer (K&A Laborgeraete GbR, Germany).
8. Ammonia solution (32 %) for oligonucleotide deprotection.
9. Urea (ultrapure).
10. 100 mL of 10× TBE buffer (tris(hydroxymethyl) aminomethane)/boric acid/EDTA.
11. 20 % polyacrylamide stock solution.
12. 10 % APS prepared by dissolution of 100 mg of ammonium persulfate (APS) in 900 μL milliQ water.
13. For denaturing gel electrophoresis: mix polyacrylamide/*N*,*N*′-methylenebisacrylamide solution (29:1 v:v), 100 mL of 10× TBE buffer, 420 g of urea, and add milliQ water to 1000 mL.
14. For a denaturing gel: mix 60 mL of 20 % stock solution, 750 μL of 10 % APS, and 30 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED).
15. Electrophoretic power supply.
16. 0.3 M triethylammonium acetate buffer used for ODN elution, obtained by mixing 300 mL of 1 M triethylammonium acetate buffer and 700 mL of milliQ water and filtrated through a MF™ Membrane filter (0.45 μm).
17. 96 % ethanol.
18. Gel filtration column (NAP™-25, GE Healthcare).
19. 100 mL of autoclaved milliQ water.
20. Dehydrated acetonitrile (max. 30 ppm of a water).
21. Freeze-drier, miVac Concentrator (Genevac, USA) used for lyophilization of probes.

2.5 Spotting of Modified ODNs

1. 40 μM modified ODNs solution.
2. 0.1 M sodium ascorbate solution, 4 mg of sodium ascorbate dissolved in 120 μL autoclaved milliQ water.
3. 0.1 M $CuSO_4$ solution, 6.2 mg of $CuSO_4 \cdot 5H_2O$ dissolved in 250 μL autoclaved milliQ water.
4. 10 mL glycerol.
5. 384-well plate.
6. Microarray spotter (NanoPrint LM60, ArrayIt, USA).

2.6 Hybridization

1. 1 % sodium dodecyl sulfate (SDS) solution: 5 g of SDS dissolved in 80 mL of milliQ water. Next, the solution was heated to 60 °C and stirred until all SDS was dissolved. The volume was adjusted to 550 mL with milliQ water and stirred again at room temperature.
2. milliQ water, $T = 90–100$ °C.
3. 20× SSC buffer: dissolve 173.5 g of NaCl and 88.2 g of sodium citrate in 800 mL of milliQ water. Adjust the pH value to 7.0 with a few drops of 14 M HCl. Adjust the volume to 1 L with milliQ water. Sterilize by autoclaving. The final concentrations are 3.0 M NaCl and 0.3 M sodium citrate [39].
4. 2× SSC + 0.1 % SDS: 50 mL of 20× SSC dilute with 300 mL milliQ water and while stirring add 50 mL of 1 % SDS. Adjust the volume to 500 mL with milliQ water.
5. 0.5 % sodium dodecyl sulfate (SDS) solution: 50 mL of 1 % SDS solution was placed in a graduated cylinder and the volume was adjusted to 500 mL with milliQ water.
6. 0.05 % sodium dodecyl sulfate (SDS) solution: 0.5 % SDS solution was diluted ten times with milliQ water in a graduated cylinder and the volume was adjusted to 500 mL.
7. Mixture of modified ODNs (2 nM, 1 μL) in 50 μL SpotQC buffer (Integrated DNA Technologies).
8. Microarray High-Throughput Wash Station (ArrayIt).
9. Microarray High-Speed Centrifuge (ArrayIt).
10. MicroarrayHybridization Chamber (Corning).
11. Microscope coverslips 24 × 24 mm.
12. MicroarrayHybridization Oven (Thermo Scientific).

2.7 Scanning and Analysis

1. Microarray scanner *GenePix 4200AL* (Molecular Devices).
2. *GenePixPro 6.0* software (Molecular Devices).

3 Methods

3.1 Glass Slide Preparation

1. Place five glass slides in a Coplin staining jar with 40 mL of acetone and sonicate for 10 min (*see* **Note 3**).
2. Dry glass slides under nitrogen gas for 20 min (*see* **Note 4**).
3. Place glass slides in detergent solution in a microarray high-throughput wash station and mix for 20 min (*see* **Note 5**).
4. Rinse twice with doubly distilled water in Microarray high-throughput wash station.
5. Dry in a stream of nitrogen gas for 30 min (*see* **Note 4**).

3.2 CPG Silanization

1. Place 0.1 g CPG and 5 % solution of AzPTMS in toluene in a round-bottom flask. Stir the mixture at room temperature for 24 h (*see* **Note 6**).
2. Filter the silanized CPG using a funnel and wash with 100 ml of toluene.
3. Place the silanized CPG in an oven and dry at 120 °C for 1 h (*see* **Note 7**).

3.3 Glass Slide Silanization

1. Immerse cleaned glass slides in Coplin staining jars containing a toluene solution of the 3-AzPTMS at various concentrations (0.02; 0.2 or 0.5 %) at room temperature for 1 h.
2. Rinse silanized glass slides by sonication in toluene (two times using 40 mL).
3. Dry in a Microarray High-Throughput Wash Station oven at 120 °C for 1 h.
4. After cooling to room temperature, rinse the glass slides with ethanol and dry in a stream of nitrogen.

3.4 Synthesis of Modified Oligodeoxynucleotides (ODN)

1. Use 4-pentyl-1-ol, diisopropylamine, 3-hydroxypropionitrile, PCl_3, and dried benzene (as a solvent) to prepare the linker: 4-pentynyl-(2-cyanoethyl)-N,N-diisopropyl phosphoramidite according to the method of *Pourceau* et al. [40].
2. ODNs were synthesized by following the protocol described by *Caruthers* et al. [3, 7]. The linker was dissolved in a 1 mL of dry acetonitrile (0.2 M) and attached to the 5′ end of each ODN during its automatic ODN synthesis on the DNA synthesizer (*see* **Note 8**).
3. Prepare 20 % polyacrylamide gel for electrophoresis (*see* Subheading 2.3, **item 11**).
4. Run the gel electrophoresis using the settings of 450 V, 10 mA, 25 W.
5. Elute ODNs from a denaturing gel with 0.3 M triethylammonium acetate buffer in two steps. First, crush the gel. Then, swamp it with 1 mL of buffer, shake it overnight at 4 °C, centrifuge, and decant the solution (part I). Put in 1 mL of buffer, shake for 4 h, centrifuge, and decant the solution (part II).
6. Put together solutions from part I and II and add three volumes of 96 % ethanol, keep it for 1 h at 4 °C, centrifuge at 18,514 × *g* force for 30 min. Decant the supernatant and wash the precipitate with 500 μL of 70 % ethanol solution. Centrifuge it at 18,514 × *g* force for 15 min at 4 °C, decant the supernatant, and dry the modified ODN precipitate.
7. The modified ODNs are desalted. Dissolve the ODNs obtained from gel in 500 μL autoclaved milliQ water. Introduce them on

a NAP™ -25 column, and elute the ODN with autoclaved milliQ water. Lyophilize the ODNs using a freeze-drier afterwards.

3.5 Spotting of Modified ODNs

1. Mix in a tube: 250 μL $CuSO_4$ solution (0.04 M), 1 μL of modified ODN (40 μM), 50 μL of glycerol, and 120 μL sodium ascorbate (0.1 M) (*see* **Note 8**).
2. Place 20 μL of the mixture into a single well of 384-well plate. In total, 100 wells per modified ODN were filled with the mixture (5 columns × 20 rows). Apply the microarray spotter pin configuration: four pins in a row (*see* **Note 9**).
3. Incubate the slides for 1 h in the spotter humidity chamber (70–80 %) at room temperature (*see* **Note 10**).
4. Using the modified ODNs in the 384-well plate, perform spotting on the slides.

3.6 Hybridization

1. Immerse the spotted slides in the High-Throughput Wash station filled with 1 % SDS solution (500 mL) at room temperature and stir for 1 min.
2. Next, fill the station with milliQ H_2O (500 mL, T = 90–100 °C) and immerse the slides in it and stir for 30 s.
3. Take out the slides and dry them by centrifugation (10 s) (*see* **Note 11**).
4. Place the slides in the MicroarrayHybridization Chamber. Place 50 μL mixture of fluorescently labeled ODN in SpotQC buffer on the slide. Cover the slide surface with coverslip and place 10 μL of milliQ H_2O in the well in the Microarray Hybridization Chamber (*see* **Note 12**).
5. Lock the MicroarrayHybridization Chamber and incubate it for 30 min at 40 °C.
6. Next, remove slides from Hybridization Chambers and immerse it in the High-Throughput Wash station filled with 2× SSC + 0.1 % SDS solution (500 mL) at room temperature and stir for 1 min.
7. Fill the station with 0.5 % SDS solution (500 mL) and immerse slides in it and stir for 1 min at room temperature.
8. Lastly, wash the slides by immersing them in 0.5 % SDS solution (500 mL) with stirring for 1 min at room temperature. Then dry them by centrifugation (10 s).

3.7 Scanning and Analysis

1. To detect fluorescence of hybridized probes, scan the microarrays using a microarray scanner (*see* **Note 13**).
2. Perform quantitative analysis using the *GenePixPro 6.0* software. As a result, convert the signal intensities into numbers and save them as .gpr files (*GenepixPro results*). This file format is used for many programs enabling advanced microarray data analysis.

4 Notes

1. The reagents: 4-pentyl-1-ol, benzene, and DIPEA must be dried prior to use, otherwise we have to use more PCl_3, which consumes water in reaction mixture, i.e., in a small excess than in the method described by *Pourceau* et al.
2. For modified CPG, we used the 1000 Å of 5'-DMT-X-Suc-CPG where X refers to nucleotides: T, dG(iBu), dC(Bz), and dA(Bz) (where iBu and Bz are isobutyryl and benzoyl exocyclic amine protecting groups, respectively).
3. Acetone removes organic contaminants from the glass surface.
4. The glass slides are dried in a desiccator through which a stream of nitrogen gas flows.
5. The use of a detergent solution is sufficient to clean the surface of the glass slides. This removes any stains that have background fluorescence to interfere with the microarray image on the glass slide.
6. The CPG silanization process is carried out in a round-bottom flask. The flask must be shaken to keep the CPG particles in constant motion. To this end, one may use an automatic shaker or mechanical stirrer. In the latter case, it is vital to keep the stir bar off the CPG particles in order to avoid destruction of their structure.
7. Thermal curing of the film which, in cross-linking the free silanol groups, reduces the effect of hydrolysis of one or more of the siloxane linkages on the glass surface. Post-silanization curing of the substrate has been shown to improve the stability of silane films by cross-linking of free silanols.
8. The time of the coupling stage was extended to 200 s to maximize the reaction yield. The presence of linker at the 5′ end of the ODNs was confirmed chromatographically after their purification.
9. Copper (I) sulfate (formed by the reduction of copper (II) sulfate by sodium ascorbate) is present to catalyze the "click" reaction. Glycerol reduces water evaporation in small liquid volumes of the spotted droplets and enables more effective covalent attachment of probes to the solid surface.
10. While using the contact microarray spotter, it is important to optimize the contact distance between the pins and the glass slide. In this way, the solution is precisely located and spread on the slide surface. The liquid spread on the slide also depends on the hydrophilic character of the surface.
11. To enable a stable attachment of the probes on the solid surface via the "click" reaction, it is crucial to keep humidity at ~70–80 % in order to decrease evaporation of the solvent.

12. The use of centrifugation to dry the slides helps to reduce the fluorescent background of the microarray image.
13. The application of nitrogen gas, especially during hybridization, results in a reduction in the fluorescent background.

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