Specific DNA Duplex Formation at an Artificial Lipid Bilayer: towards a New DNA Biosensor Technology

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Dedicated to Prof. Dr. *Fritz Eckstein*, Göttingen

A novel technique is described which comprises a base-specific DNA duplex formation at a lipid bilayer–H_2O-phase boundary layer. Two different probes of oligonucleotides both carrying a double-tailed lipid at the 5′-terminus were incorporated into stable artificial lipid bilayers separating two compartments (*cis*/*trans*-channel) of an optically transparent microfluidic sample carrier with perfusion capabilities. Both the *cis*- and *trans*-channels are filled with saline buffer. Injection of a cyanine-5-labeled target DNA sequence, which is complementary to only one of the oligonucleotide probes, into the *cis*-channel, followed by a thorough perfusion, leads to an immobilization of the labeled complementary oligonucleotide on the membrane as detected by single-molecule fluorescence spectroscopy and microscopy. In the case of fluorescent but non-complementary DNA sequences, no immobilized fluorescent oligonucleotide duplex could be detected on the membrane. This clearly verifies a specific duplex formation at the membrane interface.

Introduction. – The analysis of genes or gene segments is currently based on DNA chips. These chips or DNA microarrays are made up of a solid carrier (usually a glass object plate), on which single-stranded DNA molecules with a known sequence are attached in a regular and dense pattern. These DNA chips are *i*) either produced by direct synthesis on the solid carrier using masks and a photo-lithographic procedure, *ii*) or prefabricated, and terminally functionalized samples of nucleic acids are chemically attached to activated surfaces by covalent bonds.

The DNA analysis involves multiple steps: *i*) preparation of the sample (extraction, PCR, *etc.*), *ii*) hybridization on the chip, *iii*) stringend washing, *iv*) detection, and *v*) bioinformatic analysis.

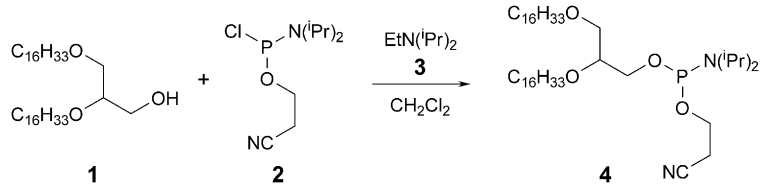
Both ways of producing DNA chips are afflicted with numerous issues. The first method requires the synthesis of oligonucleotides directly on the carrier, and includes several deprotection reactions and washing steps. This renders a complex method, especially when the array includes a multitude of different nucleic acid samples. In case of the second method, the solid surface – usually a glass plate – has to be activated with functional groups in a complicated manner in order to apply the likewise premade functionalized nucleic acids with a known sequence. This ‘spotting’ is also a challenging

procedure and requires special equipment. What follows is a chemical reaction between the ready-made functionalized nucleic acids and the activated functional groups on the surface of the array in order to achieve a covalent bond between the array and the nucleic acid [1].

Here, we present first experiments towards a novel DNA chip technology which renounces any chemistry on solid supports by using instead the self-organization and duplex formation of lipid–oligonucleotide conjugates at a lipid bilayer–water interface [2].

Results and Discussion. – Reaction of a racemate of anhydrous 2,3-bis(hexadecyloxy)propan-1-ol (**1**) with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (**2**) in the presence of *Hünig*'s base **3** afforded a diastereoisomer mixture of the phosphoramidite **4** [3] (*Scheme*).

Scheme



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This was used to prepare the oligonucleotides 5′-d(**1**-p-TAG GTC AAT ACT)-3′ (**5**) and 5′-d(**1**-p-ATC CAG TTA TGA)-3′ (**6**). Additionally, the cyanine 5-labeled oligonucleotide 5′-d[(Cy-5-p)-AGT ATT GAC CTA] (**7**) was prepared, which perfectly matches the oligomer **5** in an antiparallel strand orientation, but not the oligomer **6**. All three oligonucleotides are non-selfcomplementary, nor do they form hairpins.

All hybridization experiments were carried out with an '*Ionovation Explorer*'. This automated lipid bilayer workstation for inverted microscopes (*Fig. 1,b*) allows the combination of high-resolution microscopy with state-of-the-art electrophysiology, in particular bilayer recordings [4][5]. The oligonucleotides **5** and **6** were inserted into artificial bilayer membranes composed of 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine (POPE)/1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) 8:2 (*w*/*w*) in *n*-decane (10 mg/ml) using the microfluidic sample carrier (*Bilayer Slide*) shown in *Fig. 1,c* (for a detailed procedure description, see *Exper. Part* and [5]).

After perfusion of the *cis*- and *trans*-channels with buffer, the fluorophore-labeled oligonucleotide **7** was added to the *cis*-channel of the *Bilayer Slide*. After an incubation time of 15 min, the bilayers were optically inspected by fluorescence microscopy. Thereupon, both channels were perfused repeatedly for 30 s each (1.1 ml/min), and then the fluorescence intensity at the bilayer was measured (*Figs. 2* and *4*). From *Fig. 2*, it can be seen that, at the phase boundary, a fluorescent DNA duplex was formed after the addition of oligomer **7** to the immobilized oligomer **5** [6]. This membrane-bound duplex appears to be stable to several perfusions (30 s each; 1.1 ml/min). However, careful inspection of the *cis*-compartment and the bilayer before and after each perfusion exhibits the appearance of a low density of fluorescent nanoparticles, which are washed out upon subsequent perfusion. Simultaneously, the fluorescence intensity

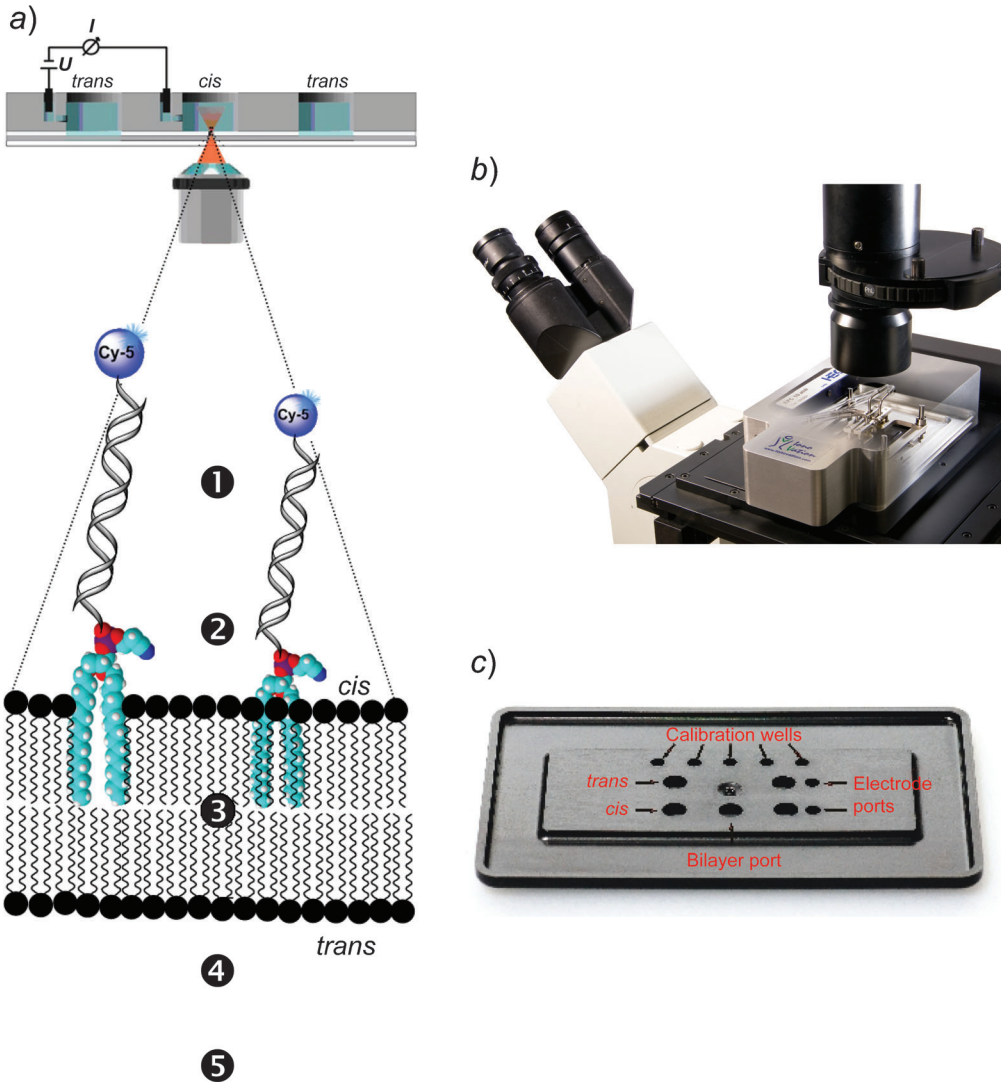


Fig. 1. *Experimental setup*. *a*) Schematic drawing of the Laser Scanning Microscope, the optical transparent microfluidic bilayer slide, and the lipid bilayer with incorporated double-tailed nucleolipids, and five measuring positions (*1*–*5*). The bilayer slide encloses two microfluidic channels (*cis* and *trans*), which are separated by a thin medical-grade PTFE (= polyterafluoroethylene, *Teflon*) foil. This foil hosts a central 100 μm aperture which is located 120 μm above the coverslip and thus within the working distance of high NA (= numerical aperture) objectives. It is the only connection between the *trans*- and *cis*-channels. When a lipid soln. is painted across the aperture, a bilayer is formed spontaneously. Electrodes in the *cis*- and *trans*-channels allow an online monitoring of the bilayer integrity, as well as electrophysiological recordings. *b*) Stage unit of the '*Ionovation Explorer*' mounted on a standard inverted fluorescence microscope. The computer-controlled perfusion unit is a side board and is not shown. *c*) '*Ionovation Bilayer Slide*'; a disposable, optical transparent microfluidic sample carrier with perfusion capabilities. The 'Bilayer Port' gives direct access to the lipid bilayer, while both sides of the bilayer can be perfused *via* the *cis*- and *trans*-channels. Calibration wells allow optical control experiments when needed.

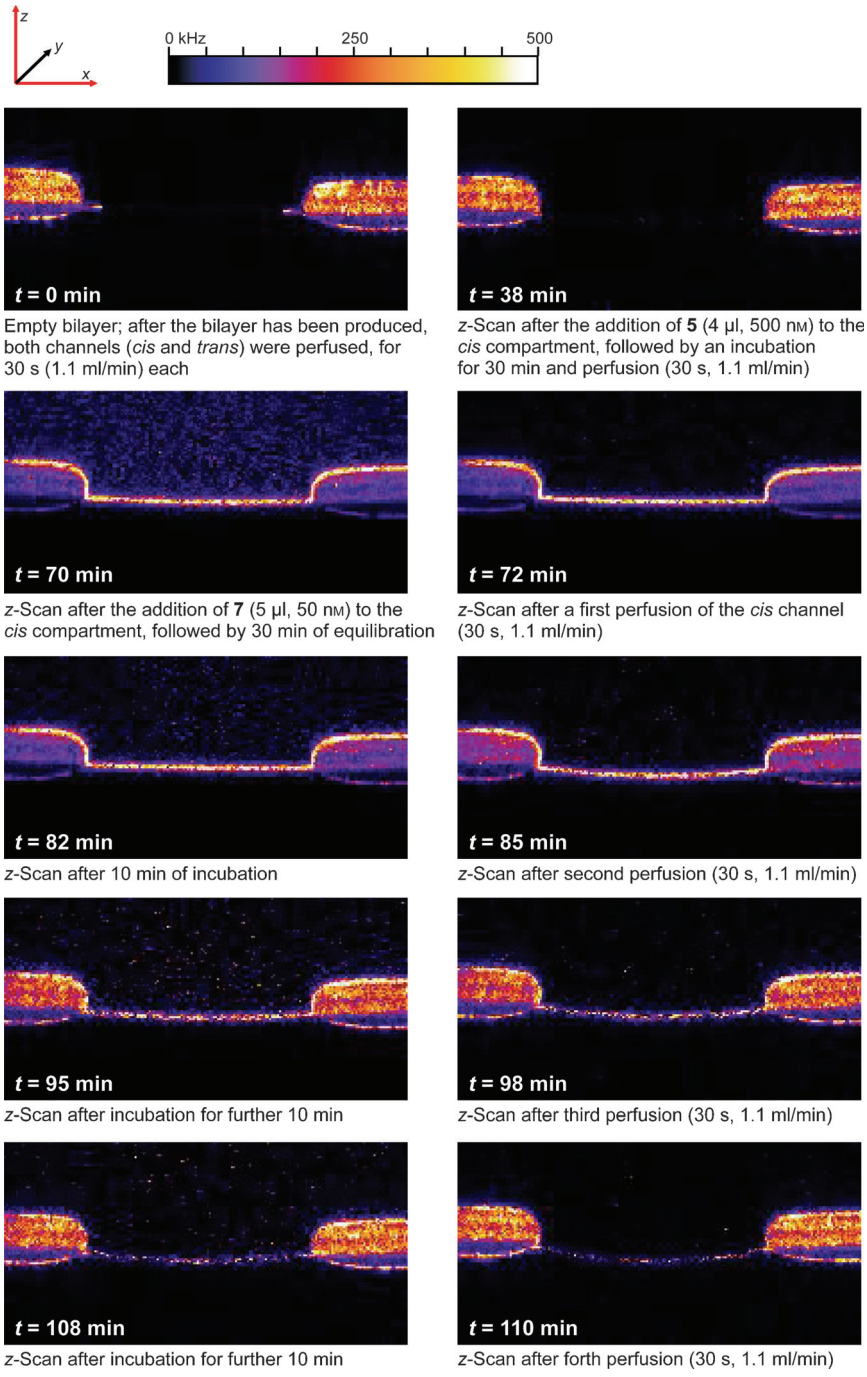


Fig. 2. *Chronological protocol* (→ ↓) *of duplex formation of the lipophilized oligonucleotide* **5** *with the complementary oligomer* **7** *at an artificial lipid bilayer–aqueous buffer boundary, followed by four perfusions*

of the bilayer drops slowly and stepwise (*Fig. 3*). We assume that the nanoparticles in the H_2O phase may consist of fluorescent micelles with the oligonucleotide duplexes protruding into the aqueous phase and the lipid residues inside. This implies an equilibrium between bilayer-bound DNA duplexes and nano-scale aggregates, which are freely diffusing within the *cis*-compartment of the *Bilayer Slide*. From *Fig. 4*, it can be seen that, upon addition of oligonucleotide **7**, which is non-complementary to the immobilized oligomer **6**, after 30 min of incubation, the whole *cis*-compartment is filled with fluorescent aggregates of the oligomer **7**, which do not concentrate at the bilayer but are perfused out from the *cis*-compartment within a few seconds.

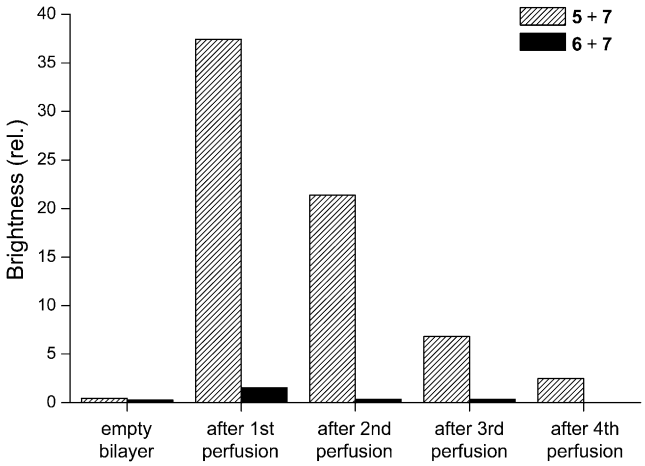


Fig. 3. *Relative bilayer brightness as a function of the perfusion number*

Furthermore, the diffusion times [ms] of the duplex **5·7** was measured, both without and in the presence of an artificial bilayer. For the determination of the free diffusion times, the corresponding oligomer duplex solution (50 nM) was diluted so that there was only a single fluorescent molecule in the confocal measuring volume (*ca.* 1 fl). Each measurement was performed ten times for 30 s. To determine the diffusion times of the lipophilized oligonucleotide duplex, **5·7**, in the presence of a bilayer two measuring positions, one above (in solution but in close proximity to the bilayer), and the other within the bilayer, were chosen. Each measurement was performed *i*) by recording reference data of a stable, blank bilayer, *ii*) after formation of the oligonucleotide duplex and a subsequent 30-min incubation, followed by recording of the data, and *iii*) recording further data series after perfusion of the *Bilayer Slide*. The results are compiled in the *Table*.

It can be seen that the diffusion of oligomer **7** as well as of the duplex **5·7** is fast. However, the broad diffusion time distribution of the duplex **5·7** indicates aggregate formation of heterogeneous size. In the close proximity of a stable bilayer, the diffusion time increases by a factor of *ca.* 10. Probably, the molecules aggregate, and the aggregates interact partly with the bilayer. The diffusion time of the bilayer-immobilized DNA duplex increases further by a factor of 10.

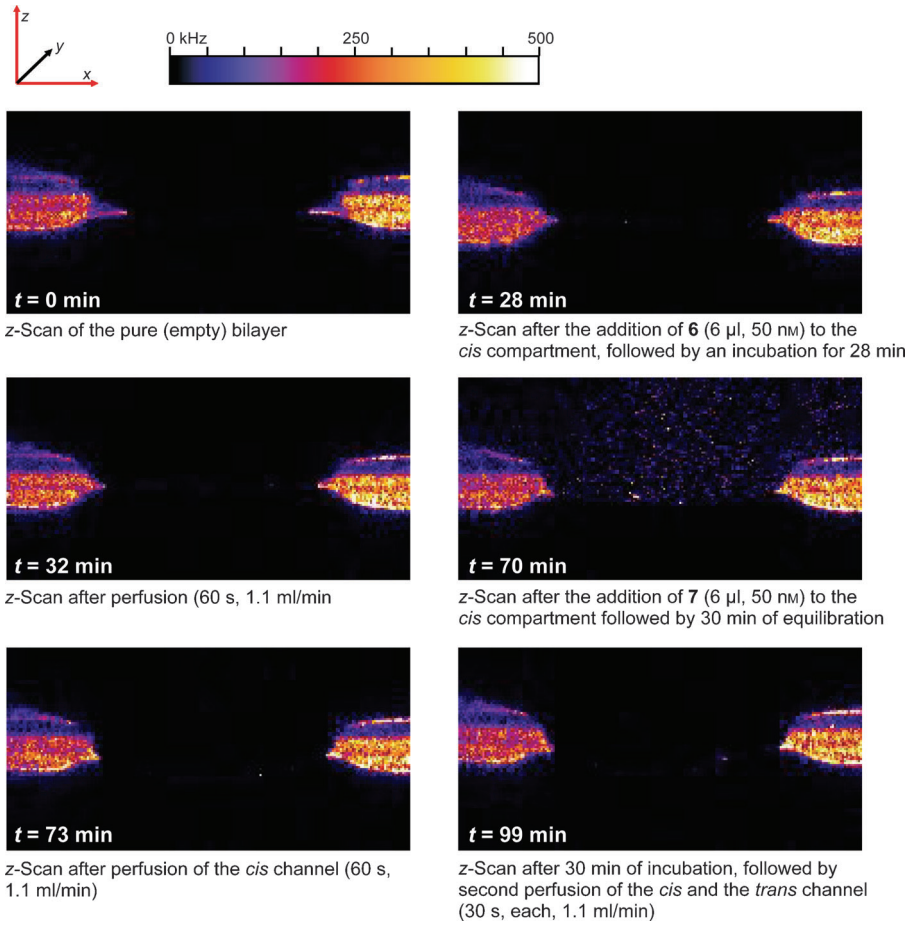
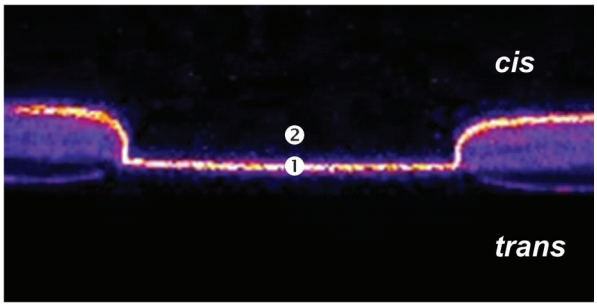


Fig. 4. *Chronological protocol* (→ ↓) *of a control experiment with non-complementary oligonucleotides,* **6** *and* **7**

Fig. 5 displays 2D as well as 3D scans of the pure and of the **5**·**7** duplex-filled bilayer. From the diffusion time measurements, a particle density of *ca.* 8 particles per µm^2 can be calculated which corresponds to *ca.* 60,000 particles within the total bilayer.

These first experiments clearly demonstrate a specific base pairing at a lipid bilayer–H_2O interface, presenting a simple method for evidencing the presence or absence of a target gene fragment using tailor-made oligonucleotide–lipid conjugates without a chemical immobilization of functionalized probe oligomers at a solid surface. Further experiments concerning *i*) stability and kinetics of duplex formation of only partly matching oligonucleotides, *ii*) the role of a short oligonucleotide spacer between the lipid and the recognition element, and *iii*) a multiple compartment chamber with a common aqueous subphase, as well as a thermostated device for a suppression of nonspecific base pairing are underway [2].

Table. *Diffusion Time* (τ_D) *of* **7** *and* **5**·**7** *without and in the Presence of a Lipid Bilayer.* Position: *1*, bilayer; *2*, solution in close proximity to the bilayer.



Sample	Position	τ_D [ms]
7	Diffusion[a])	0.24 ± 0.1
5·**7**	Diffusion[a])	0.23 ± 0.1
5·**7**	*1*	17.23 ± 2.0
5·**7**	*2*	2.47 ± 0.3

[a]) In solution without bilayer.

The authors thank the *Bundesministerium für Wirtschaft* for financial support (FKZ: KF 2369401 S B9 and KF 2369501 S B9).

Experimental Part

General. 1-Palmitoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from *Avanti Polar Lipids* (Alabaster, Al). CH_2Cl_2 was distilled at ambient pressure, dried over P_2O_5, and distilled again over P_2O_5 under Ar. Et_3N was dried with KOH and distilled over CaH_2 under Ar. Benzene was distilled at normal pressure, dried with CaH_2, and distilled again over CaH_2 under Ar. Et_2O was dried with KOH and distilled over CaH_2 under Ar. EtN^iPr was dried with CaH_2 and distilled over CaH_2 under Ar. The reaction was carried out under Ar in a dry *Schlenk* flask. Silica gel for filtration was suspended in a mixture of benzene/Et_2O 9 : 1 (*v*/*v*) with 10 drops of Et_3N per 100 ml and stirred for 1 h. The column for filtration was armed with the silica gel suspension and stabilized 1 h before filtration. $CDCl_3$ was used as an internal standard in NMR: ^1H (7.26 ppm), ^{13}C (77.00 ppm).

2,3-Bis(hexadecyloxy)propyl 2-Cyanoethyl N,N-*Diisopropylphosphoramidite* (**4**)[1]). A racemic mixture of *2,3-bis(hexadecyloxy)propan-1-ol* (**1**; 0.235 g, 0.43 mol) [7] was dissolved in CH_2Cl_2 (5 ml) and concentrated on a rotary evaporator at 30°/9 Torr. This procedure was repeated two times to remove traces of H_2O. The white residue was dissolved in CH_2Cl_2 (10 ml), EtN^iPr_2 (**3**; 0.166 g, 1.29 mol)[2]) was added at once, and the colorless transparent soln. was stirred for 5 min and cooled in an ice bath[3]). 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (**2**; 0.203 g, 0.86 mol) was added dropwise during 1 min, and the mixture was stirred for further 45 min under cooling in an ice bath. The mixture was diluted with CH_2Cl_2 (30 ml), washed with cooled H_2O (10 ml, +5°), dried (Na_2SO_4), and concentrated

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[1]) In analogy to [3], but with significant modifications.

[2]) Et_3N could be used instead of EtN^iPr_2 without significant difference in the yield and content of the reaction mixture. With pyridine, another not identified product is formed.

[3]) An insignificant white precipitate of the alcohol **1** is formed in the reaction mixture, if there is not enough solvent.

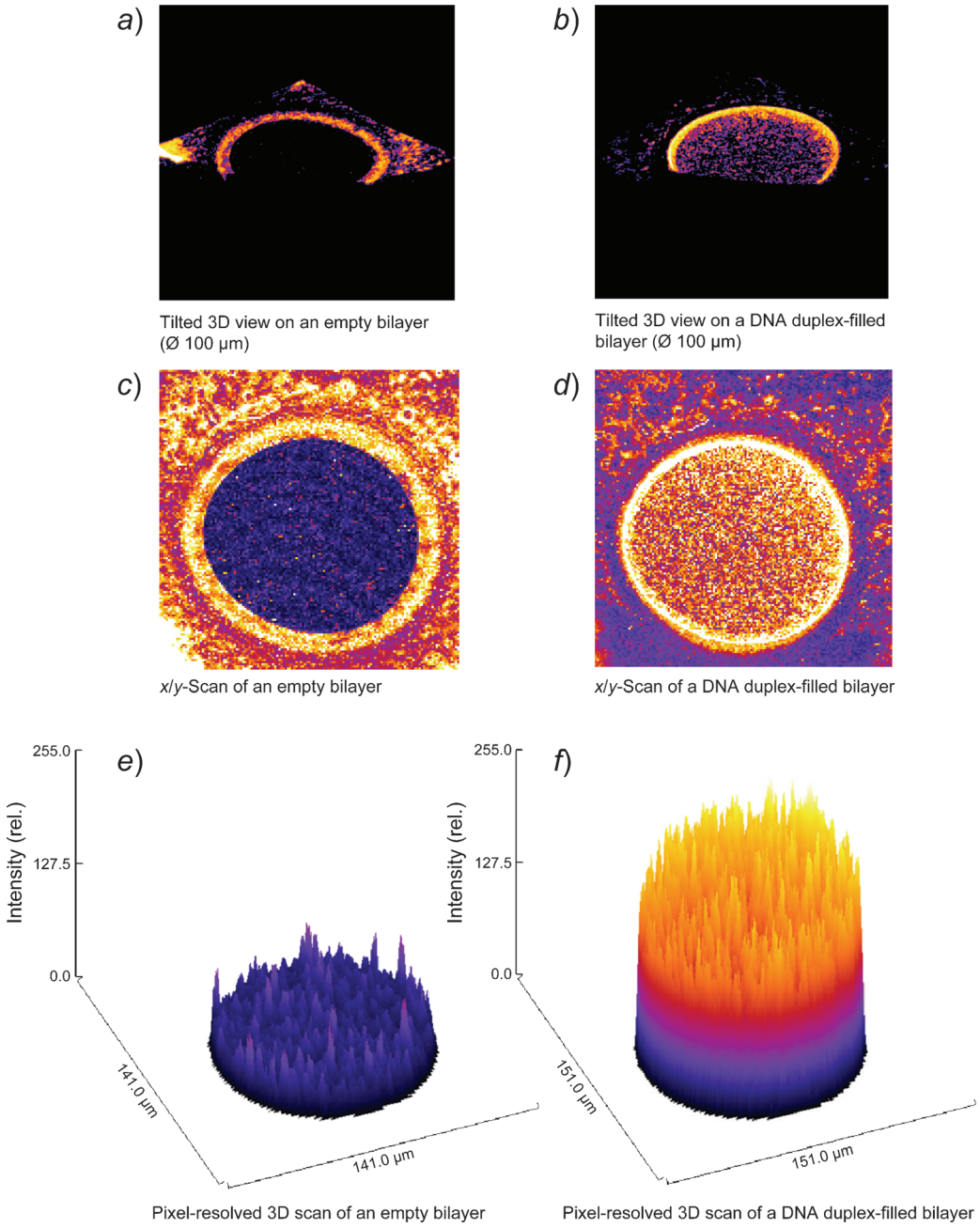

Fig. 5. *2D- and 3D scans of a pure* (empty) *bilayer and of the bilayer-inserted duplex* **5**·**7**

in vacuo (25–30°/100–10 Torr) to afford a colorless oil[4]). This oil was diluted with benzene (2 ml) and filtered through a SiO_2 layer (6-cm height), eluted with benzene/Et_2O 9:1 (*v*/*v*) with 10 drops of Et_3N per 100 ml[5]), and fractions of 0.5 times the volumes of SiO_2 were collected (one fraction within 5 min). Highly pure phosphoramidite **4** (0.18 g, 58%) in a form of a colorless oil was obtained from *Frs.* 2 and 3 upon concentration *in vacuo*. It turns into a white solid mass on cooling to 0°. The diastereoisomer mixture of **4** is stable in a pure form at low temp. (below 0°) for several months and also in a soln. of dry $CDCl_3$ at least for several days at 0°. ^{1}H-NMR (500.14 MHz, $CDCl_3$): 3.95–3.80 (*m*, CHCH_2OP); 3.71–3.44 (*m*, 11 H); 2.67–2.62 (*m*, CH_2CN); 1.57 (*sept.*, *J* = 6.9, 2 $OCH_2CH_2CH_2$); 1.24 (br. *s*, 52 H); 1.19 (*d*, *J* = 6.9, CHMe_2); 1.17 (*d*, *J* = 6.9, CHMe_2); 0.88 (*t*, *J* = 6.9, 2 CH_2Me). ^{13}C-NMR (125.76 MHz, $CDCl_3$)[6]): 117.534, 117.495 (CN); 78.493, 78.475, 78.430, 78.415 ($POCH_2CH$); 71.712 (2 $OCH_2(CH_2)_{14}Me$); 70.675, 70.622, 70.601, 70.550 ($POCH_2CHCH_2$); 63.254, 63.200, 63.128, 63.074 ($POCH_2CH$); 58.585, 58.533, 58.438, 58.386 ($POCH_2CH_2CN$); 43.222, 43.186, 43.124, 43.088 (2 NCH); 31.918 (2 $MeCH_2CH_2$); 30.148, 30.143 (2 $OCH_2CH_2(CH_2)_{13}Me$); 29.693, 29.648, 29.520, 29.346 (central CH_2 of lipid chain); 26.160, 26.119 (2 $OCH_2CH_2CH_2(CH_2)_{12}Me$); 24.643, 24.583, 24.527 (2 CHMe_2); 22.669 (2 $O(CH_2)_{14}CH_2Me$); 20.375, 20.325 ($POCH_2CH_2CN$); 14.070 (2 $O(CH_2)_{15}Me$). ^{31}P-NMR (101.3 MHz, $CDCl_3$): 148.64; 148.48.

Oligonucleotides. The synthesis of the oligonucleotides **5**–**7** and their mass spectrometric analysis were performed by *Eurogentec S. A.*, Liège Science Park, Belgium. MALDI-TOF-MS (*m*/*z*): 4247.7 (**5**, $[M+H]^+$; calc. 4247.4); 4248.1 (**6**, $[M+H]^+$; calc. 4247.4); 4178.4 (**7**, $[M+H]^+$; calc. 4178.1).

Oligonucleotide Incorporation into Artificial Bilayers. The incorporation of the oligonucleotides **5** and **6** into artificial lipid bilayers was performed using a lipid mixture 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine (POPE)/1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) 8:2 (*w*/*w*, 10 mg/ml of decane). The horizontal bilayers were produced automatically within the '*Bilayer Slides*' using an '*Ionovation Explorer*' (*Ionovation GmbH*, D-Osnabrück). After pre-filling with buffer (250 mM KCl, 10 mM MOPS/*Tris*, pH 7), the '*Bilayer Slide*' is inserted into the stage unit of the '*Ionovation Explorer*'. The Ag/AgCl electrodes are mounted, and after addition of 0.2 µl of POPE /POPC to the *cis*-compartment, the automated bilayer production was started. For the '*Ionovation Explorer*', a modified painting technique was used, where the air–H_2O interface paints the lipid across the aperture. The bilayer formation is monitored *via* capacitance measurements. When a stable bilayer is established (> 50 pF), the corresponding oligonucleotide soln. (**5**, 500 nM, 4 µl; **6**, 500 nM, 6 µl) was injected into the *cis*-compartment of the '*Bilayer Slide*'. During the incubation time of 30 min, the bilayer integrity was monitored by the '*Ionovation Explorer*' through continuous capacitance measurements.

A confocal laser-scanning microscope (*Insight Cell 3D*, *Evotec Technologies GmbH*, D-Hamburg), equipped with a 635-nm emitting laser diode (*LDH-P-635*, *PicoQuant GmbH*, D-Berlin), a 40× water-immersion objective (*UApo 340*; 40×; NA (numerical aperture), 1.15; *Olympus*, Tokyo, Japan), and an *Avalanche* photodiode detector (*SPCM-AQR-13-FC*, *Perkin-Elmer Optoelectronics*, Fremont, CA, USA), was used for the optical measurements. Fluorescence irradiation was obtained with an excitation laser power of 200±5 µW. 2D and 3D scans were performed by scanning the confocal laser spot in *xy* direction with a rotating beam scanner and movement of the objective in *z* direction. The movement in all directions was piezo-controlled which allows a nanometer precise positioning. For the 2D pictures (*z*-scans, *Figs. 2*, *3*, and *5*), the confocal plane was moved in 100-nm steps.

From the fluorescence signals of single molecules which pass the laser spot, the diffusion constants can be calculated by means of fluorescence correlation analysis. To determine the diffusion times of the fluorescent oligonucleotides within and in the proximity of the bilayer, they were measured at overall five different positions above, below, and within the layer (*Fig. 1*, *a*). At each point, five 30-s measurements, were conducted. In summary, each measuring protocol was as follows: *i*) a reference scan of the stable (empty) bilayer; *ii*) addition of the sample with 30 min of incubation, followed by a scan series; *iii*) additional scan series, each after a 1st, 2nd, 3rd, and 4th perfusion (60 s, each).

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4) The concentrated reaction mixture consists of the phosphoramidite **4** and a hydrolysis product without ^{i}Pr groups in a ratio of *ca.* 1:1.

5) There is no chance to obtain the pure phosphoramidite by silica-gel filtration or chromatography without Et_3N in the eluent.

6) Not all of the ^{13}C-NMR resonances of the 2^2 diastereoisomers could be resolved.

Subsequently, the cyanine 5-labeled oligonucleotide **7** (50 nM, 6 µl) was injected into the *cis*-compartment of the 'Bilayer Slides' containing either membrane-bound **5** or membrane-bound **6**. After an equilibration time of 30 min, the *cis*-channel was perfused repeatedly for 30 s (1.1 ml/min), and the bilayers were inspected by confocal fluorescence microscopy.

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Received September 12, 2011