



BIOASSEMBLER

Integrating bio-inspired assembly
into semiconductor manufacturing
technology for biosensors

D4.4 Optimized full-wafer DNA zipcode synthesis

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Authors: Gisela Ibáñez-Redín, Ghada Elsayed, Mathias Reisbeck

Contact Person: Mark Somoza
mark.somoza@univie.ac.at



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1.0	Draft 1	24.2.2026	Gisela Ibáñez-Redín /UNIVIE	First draft version
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List of contents

1. Background and objectives.....	4
2. Results.....	4
3. Conclusions and outlook.....	16
4. References	17

1. Background and objectives

The objective of the BIOASSEMBLER project is the development of an assembly technology for the scalable manufacturing of silicon-based, label-free, multiplexed biosensors using semiconductor fabrication platforms. The bio-inspired assembly approach relies on orthogonal DNA sequences (zipcodes) to selectively guide the immobilization of bioreceptors attached to their complementary sequences. While surface immobilization in multiplexed biosensors is typically achieved using dispensing methods, our approach aims to synthesize the zipcode sequences in situ directly on the array using nucleic acid photolithography, a technique extensively applied in microarray fabrication.

Translating this technology to large-scale biosensor fabrication requires addressing three main challenges: (1) developing optical exposure systems compatible with large-area fabrication, (2) designing and optimizing flow cells to deliver all reagents and chemicals required for the reactions to the wafer surface, and (3) optimizing chemical conditions for large-area synthesis on substrates other than glass. While most of these topics have been addressed individually in Deliverables 4.3, 3.5, and 4.1, respectively, this deliverable focuses on the integration of the photolithographic exposure system with a wafer-level reagent delivery system to synthesize DNA zipcodes directly on the sensor surfaces, which is the objective of task 4.4.

In this deliverable, we present the approaches adopted to achieve wafer-level photolithographic synthesis of DNA zipcodes on wafers featuring the BIOMEMSIO chip layout. The experiments were conducted at UniVie, and the final adaptations of the large-area flow cell developed by JOBST and the optical exposure system developed by VTT to enable integrated synthesis are described. We also discuss the optimization of chemical conditions compatible with the sensor materials, the flow cell, and large-area operation.

2. Results

2.1 Optimized large area fluid delivery

The large area flow cell that is reported in Deliverable 3.5 was further optimized for the operation with the peripheral fluidics at project partner UniVie. The large area flow cell reported is based on a silicone sealing that is permanently bonded to a glass substrate with inlet and outlet ports and the fluidic sealing is achieved using clamping of the flow cell to the substrate to be functionalized (Figure 1).

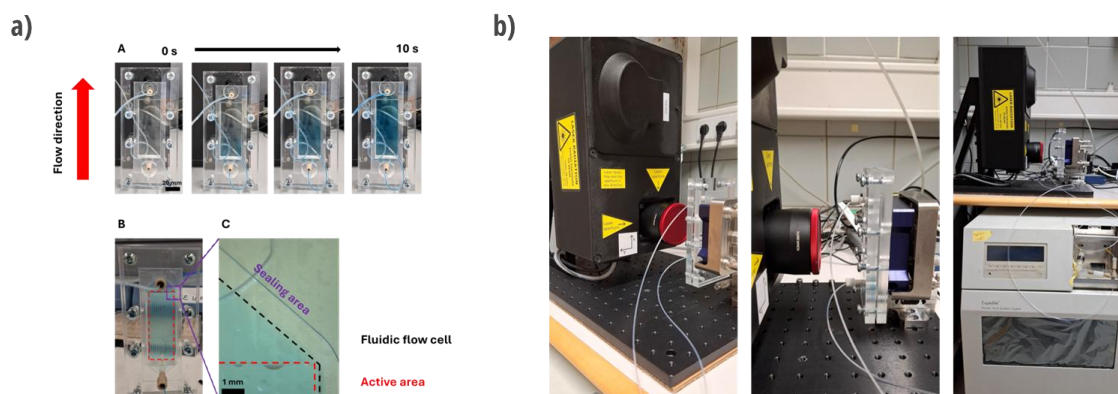


Figure 1. Initial flow cell design and results for the large area fluid delivery reported in D3.5 is based on clamping of glass substrate using a silicone seal to the wafer (a). The flow cell is compatible with the optical laser setup at the project partner UniVie and is operated in upright position (b).

The reported approach was challenging to fabricate due to the hybrid glass/silicone construction and the clamping mechanism showed stability for low pressures (< 50 mbar) that is not compatible with the fluidics setup used for the final wafer scale synthesis. This setup relies on the Expedite 8909 nucleic acid synthesizer for fluid delivery and control.

For the final device a monolithic flow cell is cast from silicone and reversibly bonded to the wafer substrate. In detail, the flow cell dimensions are based on cutting an adhesive foil with a thickness of $160\ \mu\text{m}$ followed by lamination to a PMMA substrate. The chosen dimensions of the flow cell balance sensor yield, reagent consumption, and fluidic pressure drop across the device (Figure 2 a). The fabrication of the flow cell is detailed in Figure 2 b. First, the flow cell is cast using silicone from a mold and fully cured. Inlets and outlets are inserted using $0.85\ \text{mm}$ needles for press fit of the $1/16''$ inch tubing outer diameter used in the fluidics setup. Second, a thin layer ($\sim 50\ \text{nm}$) of uncured silicone is fabricated and the silicone flow cell is stamped to the layer leaving a thin film of uncured silicone on the sealing area. Third, the thin film serves reversible adhesion bonding to the large area wafer substrate. The final device is cured for leakage-tight sealing. Due to the use of adhesion foil the optical window does not show residues of mold fabrication from e.g. micro milling (Figure 2 c). Due to the flexible nature of silicone ceiling the flow cell shows fluidic compliance (bulging of the flow cell) when high pressure are applied during filling. This effect is minimized by stabilizing the flow cell adhesion over the complete substrate using pillar structures that are bonded to the substrate. The flow cells showed robust and leakage free fluidic operation up to $10\ \text{ml/min}$ for multiple filling and rinsing cycles and were fully compatible with the operational constraints of the Expedite 8909.

After functionalization, the flow cell can be removed by gently peeling from the substrate surface. A minimal layer of few micrometers of silicone remains outside the functional area on the substrate. This was confirmed to be compatible with downstream processes such as wafer-level flow cell assembly, capping, and dicing of the substrate.

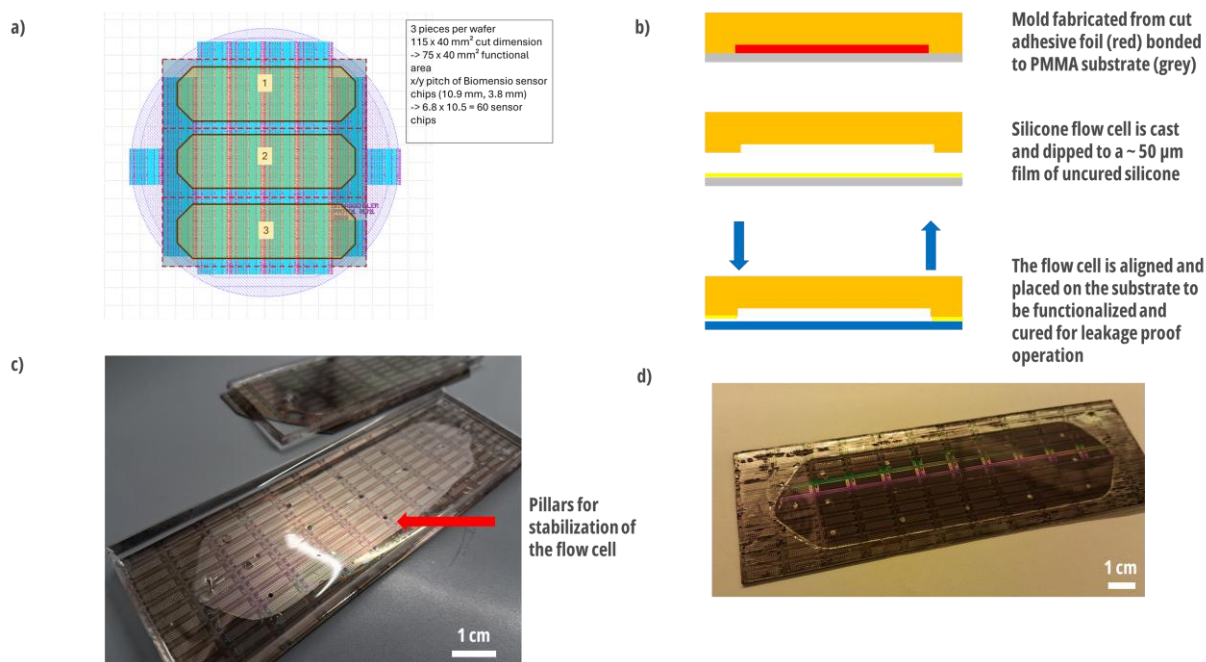


Figure 2. Top view of the large area flow cell and wafer substrate (a). An improved monolithic approach of the flow cell is fabricated and reversibly attached to the wafer substrate by adhesion bonding (b,c). The flow cell can be removed for wafer-level fluidic assembly and downstream processing of the functionalized substrate.

2.2 Second-generation laser exposure system: technical features and operational considerations for large-area DNA synthesis

In Deliverable 4.3, we addressed the feasibility of using laser scanning systems as optical exposure systems for the photolithographic synthesis of DNA. We demonstrated that DNA synthesis can be achieved using phosphoramidites bearing well-established photosensitive protecting groups (SPh-NPPOC) and short exposure times in the millisecond range when high-intensity lasers are used. However, several technical aspects of the first-generation setup required modification to ensure compatibility with wafer-level synthesis. These limitations were resolved in the second-generation laser exposure device (Figure 3 a). In addition to incorporating a higher-quality 180 mW 405 nm laser and a 2-axis fast steering mirror, the upgraded system includes a holographic beam shaper that converts the Gaussian beam profile into a square top-hat profile, enabling homogeneous illumination of the sensor surface. The system further integrates two interchangeable scanning lenses: an $F = 55$ mm lens used for small-area characterization and optimization studies, enabling scan fields of up to 20×20 mm, and an $F = 175$ mm lens for large-area synthesis, allowing synthesis areas of up to 110×110 mm to be addressed. An additional optical component enables adjustment of the spot size within a range of approximately $100\text{--}700$ µm (depending on the selected lens configuration), allowing precise control over feature dimensions. Since the BIOMEMSIO array sensor size is 200×200 µm, the zoom

setting 1.0 with the $F = 175$ mm lens was selected for large-area synthesis, corresponding to a spot size of approximately 350×350 μm (Figure 3 b). The use of a slightly larger spot size facilitates alignment and ensures accurate targeting of the sensors.

For the synthesis, exposure parameters, i.e., laser power and exposure duration, as well as the synthesis layout, are provided as CSV files containing the coordinates of the synthesis pattern and the corresponding exposure conditions. These files are read by a custom LabVIEW program that controls and directs the laser accordingly. The software also enables communication between the DNA synthesizer and the laser system. This communication is established via a data acquisition unit (DAQ), which detects a trigger signal sent by the synthesizer at the beginning of the exposure step, initiating laser irradiation (see Table 1 in section 2.3).

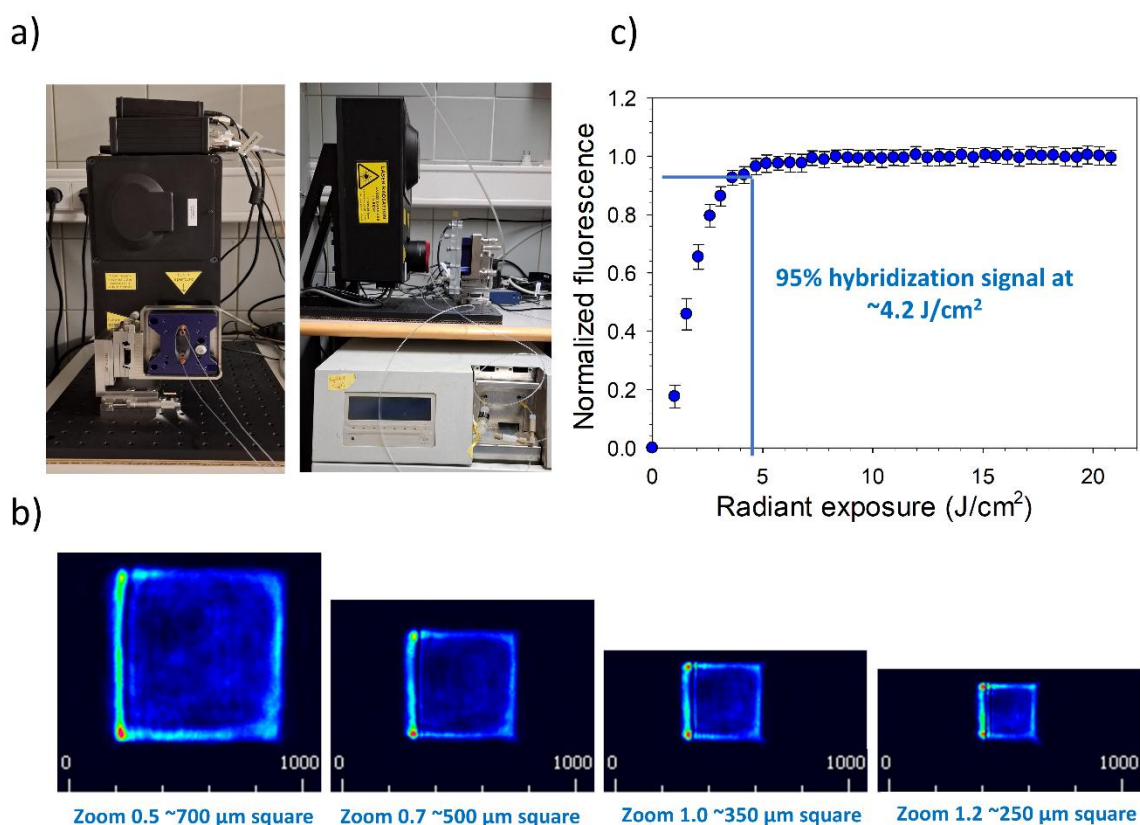


Figure 3. Photographs of the second-generation laser scanning system (a). Spot profiles at the target plane recorded with a beam camera using 0.5–1.2 zoom settings and an $F = 175$ mm scanning lens (b). Hybridization fluorescence signals of a 25-mer sequence synthesized at different radiant exposures using the laser exposure device (c).

The exposure parameters for wafer-level synthesis were established in experiments using the setup employed for glass microarray synthesis, with the optical system replaced by the laser exposure device. A radiant exposure of approximately $4.2 \text{ J}/\text{cm}^2$ was sufficient to achieve 95% of the maximum

hybridization signal, which is our benchmark for efficient photodeprotection (Figure 3 c). However, these conditions resulted in signal inhomogeneity due to the non-uniform beam profile (Figure 3 b). To ensure uniform feature illumination, a higher radiant exposure of approximately 12 J/cm^2 was selected. For the lens and zoom settings selected for the synthesis, this corresponds to an exposure duration of 106 ms using the maximum laser power. This time was further adjusted when necessary to account for differences in the optical transparency of the synthesis flow cell (see Section 2.4).

Beam distortions and positional deviations from the intended coordinates were observed during laser scanning. To achieve accurate beam positioning on the sensor surfaces, these deviations were corrected using a three-step correction procedure:

- (1) *Interpolation-based large-area correction:* A global coordinate correction was implemented using an interpolation-based calibration method. A calibration matrix correlating intended and measured positions was generated by exposing a regular pattern ($51 \times 101 \text{ mm}$, 0.5 mm step size) onto radiochromic film. The measured positions were compared with the intended coordinates to construct the correction matrix. A two-dimensional interpolation algorithm was then applied to adjust the synthesis CSV files, providing corrected coordinates for accurate laser targeting. Figure 4 a shows the distortion matrix pattern generated on the radiochromic film. Figure 4 b shows the corrected layout of the BIOMEMSIO sensor array obtained using this method. The array consists of 4×16 sensors with a pitch of $500 \mu\text{m}$.
- (2) *Array layout specific correction:* As shown in Figure 4 b, the interpolation-based correction effectively removed most large positional errors, resulting in well-aligned array layouts. Some distortions remained in the central region due to optical scanning artifacts that cannot be fully corrected with this method. However, larger deviations, such as the widening observed in the upper region, were successfully corrected. An additional correction step was introduced to address remaining local deviations, particularly along the vertical axis. This correction was applied specifically to the synthesis layout by measuring for each position, the difference between the intended and actual location. These remaining errors were compensated in the exposure files.
- (3) *Specific fine tuning:* After the array layout specific correction, the layout was further fine-tuned by projecting it onto radiochromic film. The measured positions were compared with the intended wafer layout, and any remaining deviations were corrected by adding or subtracting the residuals at each position.

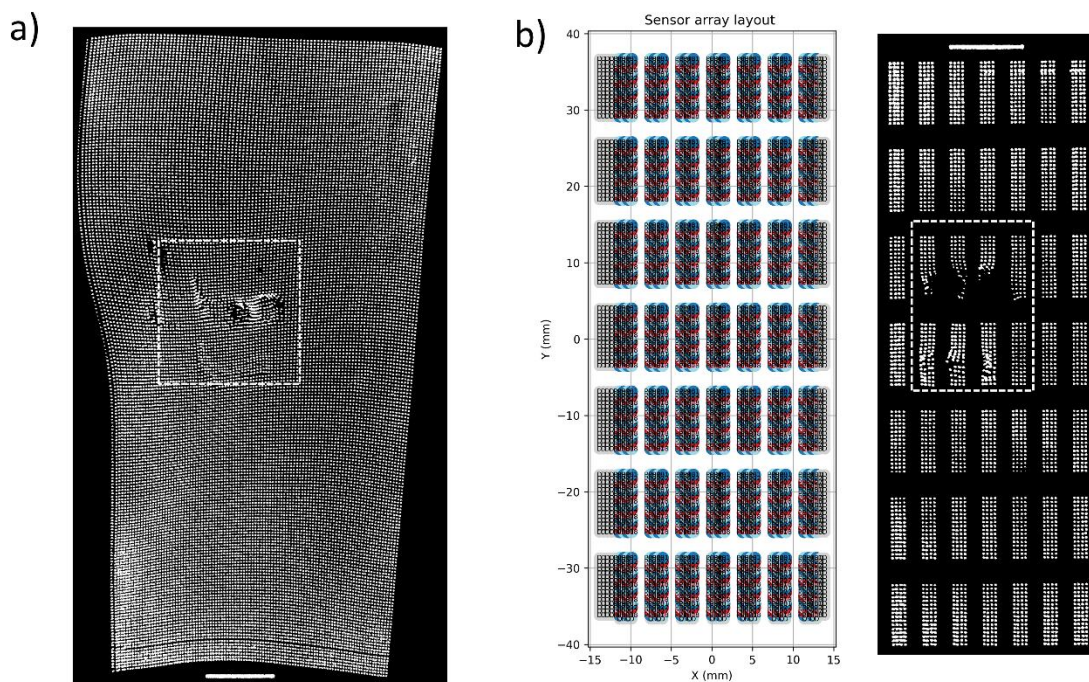


Figure 4. Pattern exposed on radiochromic film for the construction of the distortion matrix used for global correction (a). The exposure was performed using intended coordinates covering 51×101 mm with a 0.5 mm step size. Sensor array layout used for synthesis, covering 49 chips, and the corresponding layout projected onto radiochromic film after global correction (b). White squares indicate regions with scanning artifacts that could not be corrected using this approach. Scale bar: 10 mm.

All the synthesis coordinates used for the wafer-level synthesis described in section 2.4 were generated by following this procedure.

2.3 Synthesis chemistry compatible with large area fluid delivery

The chemical requirements for photolithographic DNA synthesis on glass have been extensively investigated and are well documented in the literature^{1–3}. The process begins with a surface previously functionalized to introduce reactive groups for the attachment of the first phosphoramidite. Silanization to introduce hydroxyl functionalities is most commonly employed for glass substrates, although amino- and epoxy-functionalized surfaces have also proven effective⁴. Phosphoramidite monomers are coupled to surface hydroxyl groups (or to the previously incorporated nucleotide) in the presence of an activator. Commonly used activators include dicyanoimidazole (DCI), 5-ethylthio-1H-tetrazole (ETT), and 5-benzylthio-1H-tetrazole (BTT). Coupling times as short as 15 s with 0.25 M DCI have consistently yielded high synthesis efficiencies.

A capping step may be introduced to block unreacted hydroxyl groups following coupling. Although often unnecessary for routine synthesis of short sequences, brief exposure to acetylating capping

reagents can be advantageous for the preparation of long libraries, as it reduces sequence fragmentation associated with O⁶-phosphitylation of guanine⁵. In contrast to conventional solid-phase synthesis, surface-based synthesis on glass benefits from a short (approximately 15 s) drying step under helium prior to oxidation.

The oxidation step converts the unstable phosphite triester (P(III)) linkage formed during coupling into the stable phosphate triester (P(V)) linkage. This is typically achieved by incubating for about 10 s with a commercially available iodine oxidizing solution (tetrahydrofuran/water/pyridine/iodine, 90.54/9.05/0.41/0.43 v/v/v/w) at each cycle. The final step of each cycle is photodeprotection, in which the photosensitive protecting group is selectively removed by UV irradiation. This reaction requires the presence of a weak base in the exposure solvent to facilitate efficient deprotection. We routinely use 1% of imidazole in dimethyl sulfoxide (DMSO) as exposure solvent, but other bases like piperidine, diisopropyl-ethylamine, and 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU) in acetonitrile have also proven to be efficient⁶.

In Deliverable 4.1, we described the optimized chemical conditions and surface functionalization strategies required to achieve efficient photolithographic DNA synthesis on wafer-based sensors, while ensuring full compatibility between the sensor materials and the chemicals and process steps involved in synthesis. As a functionalization strategy, we selected silanization with *N*-(3-triethoxysilylpropyl)-4-hydroxybutyramide, which yields hydroxyl-terminated surfaces suitable for phosphoramidite coupling. This approach was appropriate because the top layer of the sensors consists of a 20 nm SiO₂ film deposited by plasma-enhanced chemical vapor deposition (PECVD), providing a compatible surface for silane chemistry. The main modification to the standard synthesis protocol concerned the oxidation step. The conventional iodine-based oxidizer was replaced with (1*S*)-(+)-(10-*c* amphorsulfonyl)-oxaziridine (CSO). In contrast to iodine formulations, CSO does not induce damage to metallic components of the sensor, thereby preserving device integrity while maintaining efficient oxidation⁴.

For wafer-level synthesis, the reaction conditions were reassessed to ensure compatibility not only with the sensor materials, but also with the flow cell material and its operational constraints. Solvents such as tetrahydrofuran (THF), present in commercial iodine oxidizer formulations, as well as acetylation-based capping reagents, were found to be incompatible with the flow cell, as they induced swelling of the silicone material. As previously mentioned, iodine oxidation was replaced by CSO to avoid both metal damage and solvent incompatibility. The capping step was omitted, as it is not critical for the synthesis of short sequences, but if required, commercially available capping formulations using ACN as solvent (e.g., diethyleneglycol ethyl ether (2-cyanoethyl)-(*N,N*-diisopropyl)-phosphoramidite–UNICAP) can be employed as compatible alternatives.

A critical aspect was the drying step performed before oxidation. Although the silicone flow cells adhere sufficiently to the wafers to withstand the pressure generated by the Expedite synthesizer during reagent delivery, the 10 s helium drying step led to partial detachment of the flow cell, being therefore incompatible with the wafer-level synthesis. Since the previous studies evaluating the

impact of drying on synthesis quality were conducted using iodine as the standard oxidizer, we revisited this parameter using CSO. Figure 5 a shows the hybridization fluorescence signals of a 25-mer synthesized in series on the same array. Control sequences were prepared with a 10 s helium drying step prior to oxidation, whereas this step was omitted for the comparison sequences. The results indicate that the drying step can be eliminated without compromising synthesis quality when CSO is used as the oxidizing agent. Although the reason for this behavior remains unclear, the previously observed detrimental effect of omitting the drying step with iodine may be related to solvent differences. Iodine oxidizer formulations typically employ solvents distinct from the acetonitrile (ACN) used during coupling and washing steps. In contrast, CSO is dissolved in dry ACN, which may facilitate solvent exchange and more efficient removal during washing.

Another critical parameter is the viscosity of the exposure solvent. DMSO has been routinely used for photolithographic synthesis on glass because its refractive index closely matches that of glass, therefore minimizing light reflection and scattering at surface interfaces. However, DMSO is relatively viscous, which significantly slows reagent exchange within the flow cell. For wafer-level synthesis, we hypothesized that continuous renewal of the exposure solvent during UV irradiation could be advantageous. In large-scale syntheses, higher amounts of cleaved protecting groups are generated during the photodeprotection step. These photolysis products can accumulate within the flow cell and absorb part of the incident UV light, thereby affecting the photocleavage reaction. Maintaining a constant solvent flow during irradiation would help remove these species. For this reason, we selected 1% imidazole in ACN as the exposure solvent.

Figure 5 b shows the hybridization fluorescence signals of a 25-mer sequence synthesized in series on glass, with 1% imidazole dissolved either in DMSO or in ACN. Although the use of ACN results in slightly lower fluorescence signals in small-scale synthesis, greater benefits are expected at the wafer level, where continuous solvent flow during exposure can enhance the removal of photolysis products and improve the overall deprotection efficiency.

Figure 5 c shows the final synthesis cycle established for wafer-level synthesis using SPh-NPPOC-protected phosphoramidites. The synthesis is performed entirely in dry ACN, which serves as the solvent for the synthesis reagents and for all washing steps. Commercial formulations of 0.25 M DCI (Sigma) and 0.5 M CSO (Eurogentec) were used as activator and oxidizer, respectively. SPh-NPPOC phosphoramidites (Pitsch Nucleic Acids) were used at a concentration of 0.05 M. After synthesis, the flow cell is removed, and the wafer pieces are immersed in a 1:1 mixture of ethylenediamine (EDA) and ethanol to remove the remaining cyanoethyl and base-protecting groups.

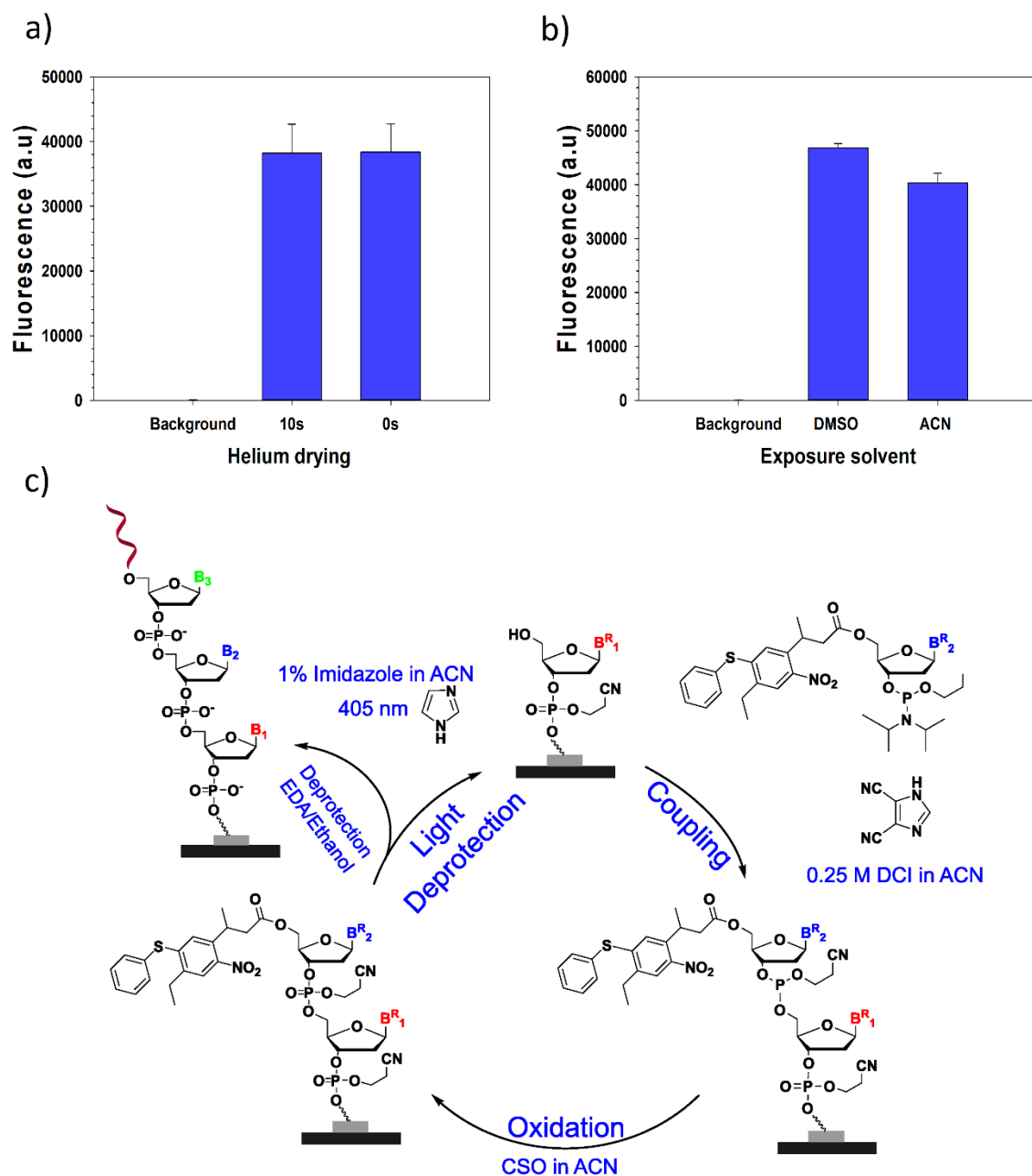


Figure 5. Optimization of the chemistry for wafer-level photolithographic DNA synthesis. (a) Hybridization fluorescence intensities of a 25-mer sequence synthesized either under standard conditions, including a 10 s helium drying step prior to oxidation, or with the drying step omitted. (b) Hybridization fluorescence intensities of a 25-mer sequence synthesized using two different exposure solvents: 1% imidazole in DMSO or 1% imidazole in ACN. (c) Chemical synthesis cycle for the wafer-level synthesis using the SPh-NPPOC photolabile protecting group.

An example protocol for a synthesis cycle, including reagent volumes and reaction times, is provided in Table 1. Slight variations in coupling, oxidation, and exposure times were evaluated and are discussed in the following section. In general, coupling times between 50 and 180 s were employed. The oxidation protocol consists of 30 s per cycle, followed by a final oxidation step of 500 or 600 s at the end of the synthesis. The duration of the exposure step varied depending on the opacity of the flow cell.

*Table 1. Protocol for wafer-level photolithographic DNA synthesis using the Expedite 8909 synthesizer as the fluidics system. Each pulse corresponds to approximately 15 μ L of reagent delivered to the flow cell. The command *130/Event 2 out triggers the laser exposure device and initiates the irradiation step. The time interval between *130/Event 2 out and the beginning of the final *12/Wsh A command corresponds to the duration of the exposure step.*

Cycle A (DNA-A)				
Function	Mode	Pulses	Sec	Description
\$Coupling				
1/*Wsh	Pulse	120	0	“Flush system with Wsh”
2/*Act	Pulse	6	0	“Act”
18/*A + Act	Pulse	22	0	“A + Act”
2/*Act	Pulse	15	0	“Chase with Act”
1/*Wsh	Pulse	15	30	“Couple monomer”
1/*Wsh	Pulse	1	20	“Couple monomer”
1/*Wsh	Pulse	120	0	“Flush system with Wsh”
\$Oxidizing				
15/*Ox	Pulse	50	0	“Ox”
12/*Wsh A	Pulse	25	0	“Ox to cell with Wsh A”
12/*Wsh A	Pulse	5	30	“Oxidize”
12/*Wsh A	Pulse	120	0	“Flush system with Wsh A”
17/*Aux	Pulse	60	0	“Aux”
130/*Event 2 out	NA	4	1	“Event 2 out”
17/*Aux	Pulse	700	700	“Aux”
12/*Wsh A	Pulse	5	60	“Flush system with Wsh A”
12/*Wsh A	Pulse	120	60	“Flush system with Wsh A”

2.4 Optimized large area zipcode synthesis

Three flow cells were evaluated for large-scale synthesis. Prior to flow cell attachment, the wafer samples were functionalized using silane chemistry, following previously described procedures⁷. In all cases, the corrected coordinates, comprising 41 active chips, were used (see section 4.2). Four DNA

zipcode probes, P01, P02, P07, and P08, were synthesized on top of a thymidine₅ linker (T₅) according to the layout shown in Figure 6 a. The CSV synthesis files, containing the corrected coordinates as well as the exposure parameters (laser power and exposure time for positions requiring deprotection) for each synthesis cycle, were generated using a custom Python script. This script optimizes the synthesis cycles and generates the flow sequence used to synchronize laser exposure with fluidic delivery. Synthesis of the four 18-mer sequences plus the dT₅ linker required 40 synthesis cycles using this optimization strategy.

For synthesis, the flow cell/wafer assembly was positioned at the appropriate working distance in front of the laser, and the beam was aligned to a reference position on the sensor array (Figure 6 b). Table 2 summarizes the synthesis conditions used for the different flow cells, as well as the total synthesis duration.

The photodeprotection step accounts for most of the total synthesis time. In addition to the exposure time required for all positions deprotected in a given cycle, extra time is needed for mirror movement between positions and for mirror stabilization after each displacement. Syntheses 1 and 2 were done using the “opaque” flow cells, which have a transmittance of approximately 50%. This reduced transmission was compensated for by increasing the exposure time, resulting in a total synthesis duration of approximately 11 hours.

Optimized synthesis conditions were achieved with flow cell 3, which, in addition to requiring a lower reagent volume, features a transparent front that allows the use of shorter exposure times. The total synthesis time was further reduced by applying two different exposure durations, depending on the number of positions exposed in each cycle. Additional optimizations included extending the coupling time for the linker to 3 min to improve the efficiency of the initial coupling steps, which are typically less efficient, and increasing the oxidation time. An additional step to empty the flow cell before introducing the amidites and oxidizer was also introduced, as this was observed to improve synthesis yields. This can be achieved, for example, by pumping from an empty port when performing syntheses with the Expedite 8909 synthesizer. Under these conditions, 41 chips were functionalized in a total synthesis time of 7 h 40 min. Figure 6 c shows the functionalized wafer after 12 h of deprotection in EDA:ethanol, displaying the characteristic hydrophilic behavior of DNA-functionalized surfaces.

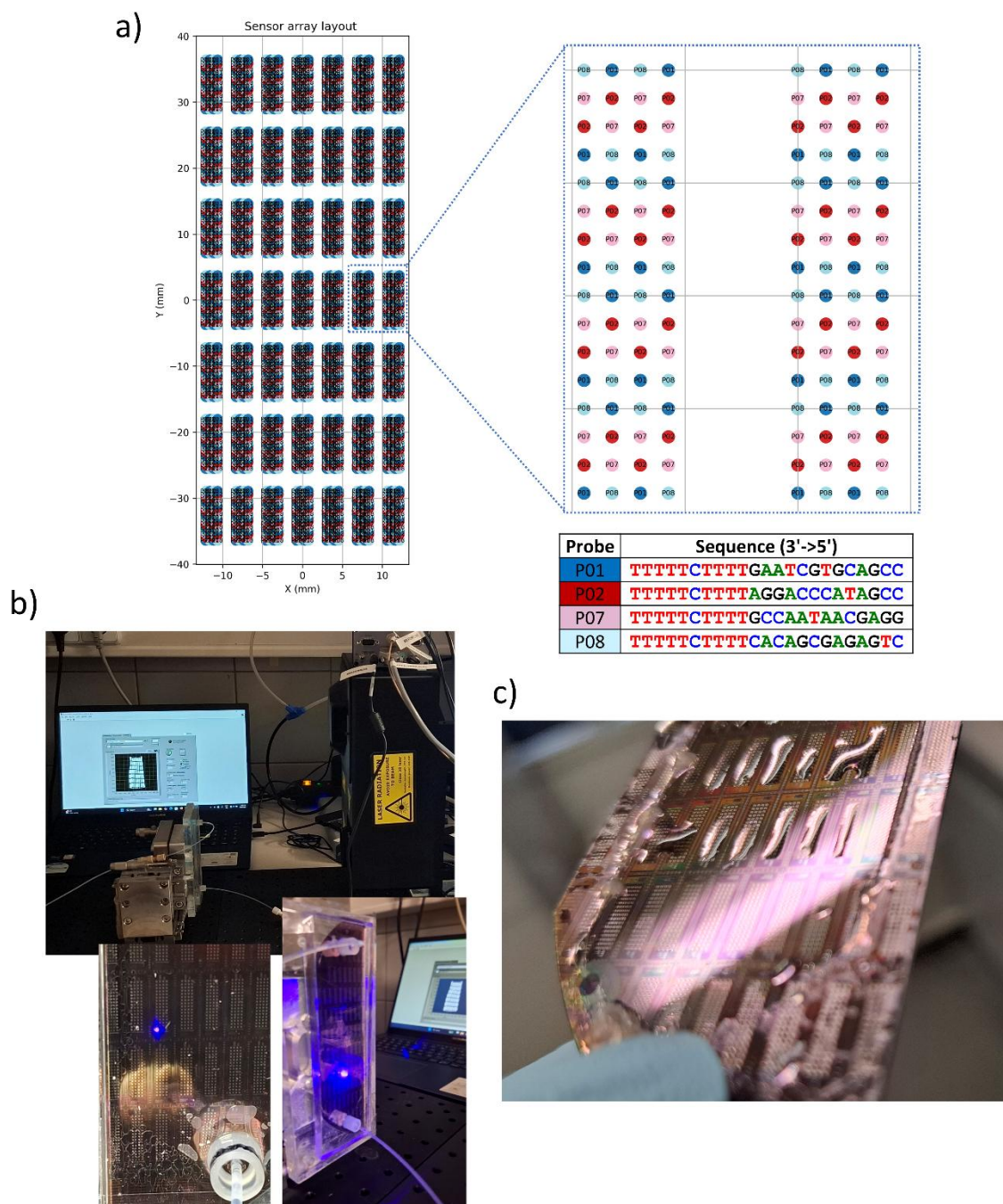


Figure 6. Layout used for the photolithographic synthesis of the DNA zipcodes on the sensors (a). Photographs showing the positioning of the flow cell in front of the laser and alignment of the laser beam with the array (b). Photograph of a functionalized wafer sample after deprotection in EDA:ethanol. The surface of the biofunctionalized sensors appears significantly more hydrophilic due to the presence of DNA (c).

Table 2. Conditions and parameters for the wafer-level photolithographic zip-code synthesis.

Cell	Cell volume	Coupling time linker	Coupling time sequence	Oxidation protocol	Exposure time/spot	Exposure time/cycle	Synthesis duration
1	400 μ L	90 s	60 s	30 s/cycle +600 s final	213 ms	760 s	11 h 10 min
2	400 μ L	80 s	50 s	30 s/cycle +500 s final	213 ms	760 s	11 h 05 min
3	300 μ L	180 s	60 s	30 s/cycle +600 s final	107 ms	500 s or 290 s	7 h 40 min

3. Conclusions and outlook

This deliverable presents the optimized wafer-level synthesis of DNA zipcodes, combining laser-directed photodeprotection, a flow cell for large-area fluidic delivery, and reagent handling with a standard nucleic acid synthesizer. Flow cells were developed to ensure leakage-free operation under the fluidic conditions required by the Expedite 8909 nucleic acid synthesizer. The reversible silicone flow cell demonstrated robust and stable performance, supporting syntheses exceeding 11 h without leakage. Successful integration of the fluidic system with the optical exposure setup enabled an optimized protocol capable of functionalizing 41 chips within 7 h 40 min.

Synthesis conditions can be further improved to reduce processing time and increase the number of addressable sensors by overcoming technical limitations, primarily related to the laser scanning device. Scanning artifacts in the central region of the scan field resulted in the non-functionalization of eight chips aligned in this area. This limitation could be mitigated, for example, by shifting the scan area to artifact-free regions (e.g., toward the right side of the field), although this would require an extended global distortion correction covering a larger area. From a chemical perspective, only minor modifications were necessary to achieve wafer-level synthesis compared to glass microarray synthesis. The amidite concentration was increased from 0.03 M to 0.05 M to compensate for potential dilution effects due to slower reagent exchange in the large-area flow cell. Activator and oxidizer concentrations were kept at standard solid-phase synthesis conditions, while reaction times were slightly extended.

From a practical perspective, amidite consumption could be further reduced, as the optimized flow cell still requires approximately 300 μ L to fill. However, this cell was designed to meet the constraints of the UniVie setup, specifically the pulse-mode, high-pressure operation of the Expedite 8909. The flow cell reported in Deliverable 3.5 requires only 100 μ L for filling and can be integrated into custom-built fluidic systems or commercially available DNA synthesizers operating under less restrictive conditions, such as the K&A DNA synthesizer.

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