

Funding Scheme: THEME [ICT-2007.8.0] [FET Open]

## Paving the Way for Future Emerging DNA-based Technologies: Computer-Aided Design and Manufacturing of DNA libraries

Grant Agreement number: 265505

Project acronym: CADMAD

Deliverable number: **D3.9**

Deliverable name: Production of a Microfluidic chip for DNA processing steps

Contractual Date <sup>1</sup> of Delivery to the CEC: <b>M24</b>
Actual Date of Delivery to the CEC: <b>Revision May 31 2013</b>
Author(s) <sup>2</sup> : <b>Patrick Wagler, Uwe Tangen, John S. McCaskill</b>
Participant(s) <sup>3</sup> : <b>RUB</b>
Work Package: <b>WP3</b>
Security <sup>4</sup> : <b>Pub</b>
Nature <sup>5</sup> : <b>R</b>
Version <sup>6</sup> : <b>2.0</b>
Total number of pages: 22 + Appendix

<sup>1</sup> As specified in Annex I

<sup>2</sup> i.e. name of the person(s) responsible for the preparation of the document

<sup>3</sup> Short name of partner(s) responsible for the deliverable

<sup>4</sup> The Technical Annex of the project provides a list of deliverables to be submitted, with the following classification level:

**Pub** - Public document; No restrictions on access; may be given freely to any interested party or published openly on the web, provided the author and source are mentioned and the content is not altered.

**Rest** - Restricted circulation list (including Commission Project Officer). This circulation list will be designated in agreement with the source project. May not be given to persons or bodies not listed.

**Int** - Internal circulation within project (and Commission Project Officer). The deliverable cannot be disclosed to any third party outside the project.

<sup>5</sup> **R (Report)**: the deliverables consists in a document reporting the results of interest.

**P (Prototype)**: the deliverable is actually consisting in a physical prototype, whose location and functionalities are described in the submitted document (however, the actual deliverable must be available for inspection and/or audit in the indicated place)

**D (Demonstrator)**: the deliverable is a software program, a device or a physical set-up aimed to demonstrate a concept and described in the submitted document (however, the actual deliverable must be available for inspection and/or audit in the indicated place)

**O (Other)**: the deliverable described in the submitted document can not be classified as one of the above (e.g. specification, tools, tests, etc.)

<sup>6</sup> Two digits separated by a dot:

The first digit is 0 for draft, 1 for project approved document, 2 or more for further revisions (e.g. in case of non acceptance by the Commission) requiring explicit approval by the project itself;

The second digit is a number indicating minor changes to the document not requiring an explicit approval by the project.

<b>1</b>	<b>KEYWORDS .....</b>	<b>2</b>
<b>2</b>	<b>AIM AND OBJECTIVES .....</b>	<b>2</b>
<b>3</b>	<b>OVERALL DESIGN AND FUNCTIONALITY .....</b>	<b>3</b>
3.1	DNA PROCESSING STEPS.....	3
<b>4</b>	<b>IMPLEMENTATION.....</b>	<b>4</b>
4.1	CHEMICAL MICROPROCESSORS - FABRICATION .....	4
4.2	CHEMICAL MICROPROCESSORS – DESIGN AND IMPLEMENTATION .....	5
4.3	MAIN DNA PROCESSING OPERATIONS ON CHIP .....	6
4.3.1	<i>Import of droplets from MWP - combinatorial interface (Step I).....</i>	<i>6</i>
4.3.2	<i>Droplet generation and combinatorial droplet mixing (Step II) .....</i>	<i>8</i>
4.3.3	<i>On-chip DNA synthesis (Step III) .....</i>	<i>11</i>
4.3.4	<i>Product extraction from droplet into separation gel (Step IV).....</i>	<i>12</i>
4.3.5	<i>On chip separation of DNA synthesis products by feedback wave electrophoresis (Step V) .....</i>	<i>13</i>
4.3.6	<i>Product injection into droplet from hydrogel matrix (Step VI) .....</i>	<i>17</i>
4.3.7	<i>Droplet transport and selection processes on chip (Step VII) .....</i>	<i>17</i>
4.3.8	<i>Export of droplets from chip to MWP (Step VIII).....</i>	<i>17</i>
<b>5</b>	<b>CONCLUSIONS .....</b>	<b>18</b>
<b>6</b>	<b>ABBREVIATIONS .....</b>	<b>19</b>
<b>7</b>	<b>REVISION NOTES &amp; RESPONSES TO QUERIES.....</b>	<b>20</b>
7.1	ANSWERS TO SPECIFIC QUERIES .....	20
7.2	RESPONSES TO FURTHER REVIEW CRITICISMS OF D3.9 .....	20
<b>8</b>	<b>APPENDIX .....</b>	<b>21</b>
<b>9</b>	<b>REFERENCES .....</b>	<b>22</b>

## 1 Keywords

Microfluidics, MEMS fabrication, DNA processing, on-chip separation, digital droplet processing.

## 2 Aim and objectives

The aim of this deliverable is to document the design and production of microfluidic chips to implement the key processes necessary for the integration of DNA editing synthesis on the sub-nanoliter scale. For the CADMAD project, at this microscopic volume scale, this is a pilot activity, originally not envisaged to yield complete DNA libraries on the timescale of the project. The future advantages of the smaller volume scale, once mastered, are clear: it will enable, as in the familiar case of transistor miniaturization, future massive integration for spatially discrete handling of very large libraries (tens of thousands to millions of DNA sequences) with moderate resources. DNA editing, like word processing ultimately requires this economy of scale. In the project, we did not, however, apply to perform this scale up, instead placing the focus on elementary processing steps. The utility of picoliter scale DNA processing depends on the availability of efficient and accurate DNA amplification, if macroscopic copy numbers of DNA library members in larger volumes are required: and such procedures indeed exist, with PCR and bacterial cloning for example, and obviously these final macroscopic processes must be performed separately downstream in larger volumes, since these volumes are not available in microfluidic chips. The challenges of microscale synthesis are very different from the near  $\mu\text{l}$  scale, addressed either in pipetting robots like Tecan and Hamilton, as employed elsewhere in the CADMAD project, or in current commercial “millifluidics” now at the scale of 0.3  $\mu\text{l}$ , such as ALL’s “digital microfluidics” being implemented for CADMAD.

In particular, the integration of successive rounds of synthesis (e.g. multiple DNA Y operations in a hierarchical DNA synthesis strategy) requires integrated product separation from reactants and by-products at intermediate steps and so this was one main theme of the DNA processing chips developed in the first two years of CADMAD: it involves product extraction, separation and reinjection into fresh reaction volumes (sub nl). This was one major focus of design and testing of the microfluidic chips in CADMAD at RUB reported in this deliverable. We chose in the project to focus on isothermal synthesis strategies, despite the ability of other groups to do on chip thermal PCR, because of material integration limits in isolating the temperature changes required for PCR with other processes such as electrophoretic separation. This decision was taken together with the partner Weizmann, who then investigated the reliability of their chosen isothermal method of nicking SDA amplification for longer DNA synthesis in the first year of the project. At the same time and carrying on into year two, RUB established and employed a closely related nicking SDA reaction (based on the Montagne system<sup>1</sup>) for short oligomers (<100 nt) as a test system for isothermal DNA synthesis and amplification. This system does produce the desired products and emulates a Y operation and subsequent amplification. Work concentrated on scaling this system down to microscopic volumes, dealing with issues of fluorescent labelling, wall absorption and droplet inactivation.

Secondly, handling populations of droplets at this microscopic scale requires the development of combinatorial droplet generation devices: and the difficulties in managing such processes increase dramatically below the  $\mu$ l scale (the scale accessible to classical pipetting). While various spotting devices at the picoliter scale have been developed (e.g. the HP inkjet technology, currently only available for DMSO solutions), none of these is available with a direct interface to microfluidic processing. For this reason, we also developed a sub-nanoliter scale on demand droplet generator, which can create programmable droplets from eight 100nl-1 $\mu$ l-scale millifluidic droplet reservoirs. In upcoming work, these millifluidic plug droplets will be generated programmably (by pressure extraction) from 8 selected wells of a microwell plate (96, 384 or 1536), or from a millifluidic EWOD device such as that developed by ALL. This will mean that 1 nanoliter scale droplets can be created as programmable mixtures of up to any 8 solutions from a microwell plate, but it will be more efficient to create many programmed mixtures from the same 8 solutions (e.g. there are 256 binary combinations but many thousands of significantly different programmable mixtures), before moving onto the next set of 8 milliscale input droplets. Because ALL's PCB-cartridges did not allow interfacing (without the large industrial costs involved in reengineering their injection moulded top layer), RUB continued to develop its own in house EWOD system that could accommodate a customizable interface via capillaries to microfluidic chips. Several project months were assigned to RUB during 2012 for this purpose, in parallel with the negotiations with Tecan and ALL, and RUB carried through on this request by the coordinator to produce a first EWOD device. Problems with the dielectric coating layer have delayed full operability of this device, although programmable droplet movement was achieved, so that the focus has now shifted back to the 8x pressurized interface to microwell plates.

### 3 Overall design and functionality

#### 3.1 DNA processing steps

The specific processes of programmed DNA synthesis (see also figure 2) using microfluidics supported by microfluidics developed at RUB in this deliverable are (in order of overall processing):

- I. **Import** of 100 nl-1  $\mu$ l scale droplet plugs from microwell plate (96/384) into 8 input capillaries, via 8x pipetting head and wells with septa under pressure, or via custom EWOD device of RUB.
- II. On chip **mixing** of 2-6 chosen input DNA species with enzymes and buffer solutions to sub-nl scale droplets. While the classic Y operation works with just 2 dsDNA input strands, Weizmann has investigated the integration of multiple ligations in a single step using the so-called Gibson reaction, so that the larger numbers (here up to 6) of DNA combinations in single droplets are an advantage.

- III. On chip **isothermal synthesis** reaction like nSDA (nicking strand displacement amplification), C2CA (circle to circle amplification) or the Gibson reaction, requiring 20 min to 1 hr incubation, and hence local parking of droplets, out of the fast generation stream, on the microfluidic chip.
- IV. On chip **extraction** of products from droplets into gel phase channel.
- V. On chip **separation** of DNA synthesis products in the gel phase.
- VI. On chip selective **reinjection** of DNA products into new droplet for further processing or output.
- VII. Managing **droplet transport** and selection processes on chip, including allowing iterative droplet processing to facilitate multiple rounds of DNA processing.
- VIII. **Export** of droplets from chip to microwell plate dispenser.

Work has concentrated on steps (II)-(VII), but in the context of future connection (in year 3) to steps (I) and (VIII).

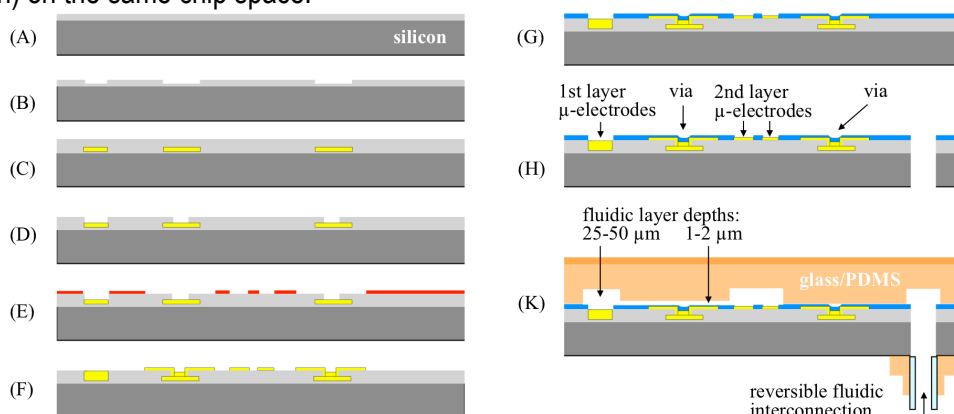
## 4 Implementation

The main thrust of this section is to describe the major implementation of functionalities for DNA editing in microfluidics. The microfluidic designs fabricated are documented systematically in the Appendix and the film material completing a demonstration of functionality is also collected there.

### 4.1 Chemical Microprocessors - fabrication

Using digital pulsed electric fields, a rather general digital field programmable control of microsystems has been developed.<sup>2</sup> Among the many known electrically inducible effects in microsystems – including electroosmosis, electrowetting, and electrochemical reactions – digital electrophoresis provides a generic control of molecular transport and concentration for charged species in channel networks. For the programmable local control of electrophoresis, fine-grained electrode arrays are required with distances allowing digital voltages to overcome thermal diffusion.

We have employed a unilayer silicon chip design, with 288 individually controllable microelectrodes (size typically  $20 \times 40 \mu\text{m}$  or  $40 \times 40 \mu\text{m}$ , 96 per module) connected to a standard field-programmable gate-array (FPGA, Spartan XCS20-CSP144 Xilinx) that serves as the driver for the electrodes and as the interface to the reconfigurable custom board. Recent developments<sup>3</sup>, progressing to a two-layer electronic chip design, allowed the integration density to increase to more than 5000 electrodes (74 blocks containing 72 individually controlled electrodes each) on the same chip space.



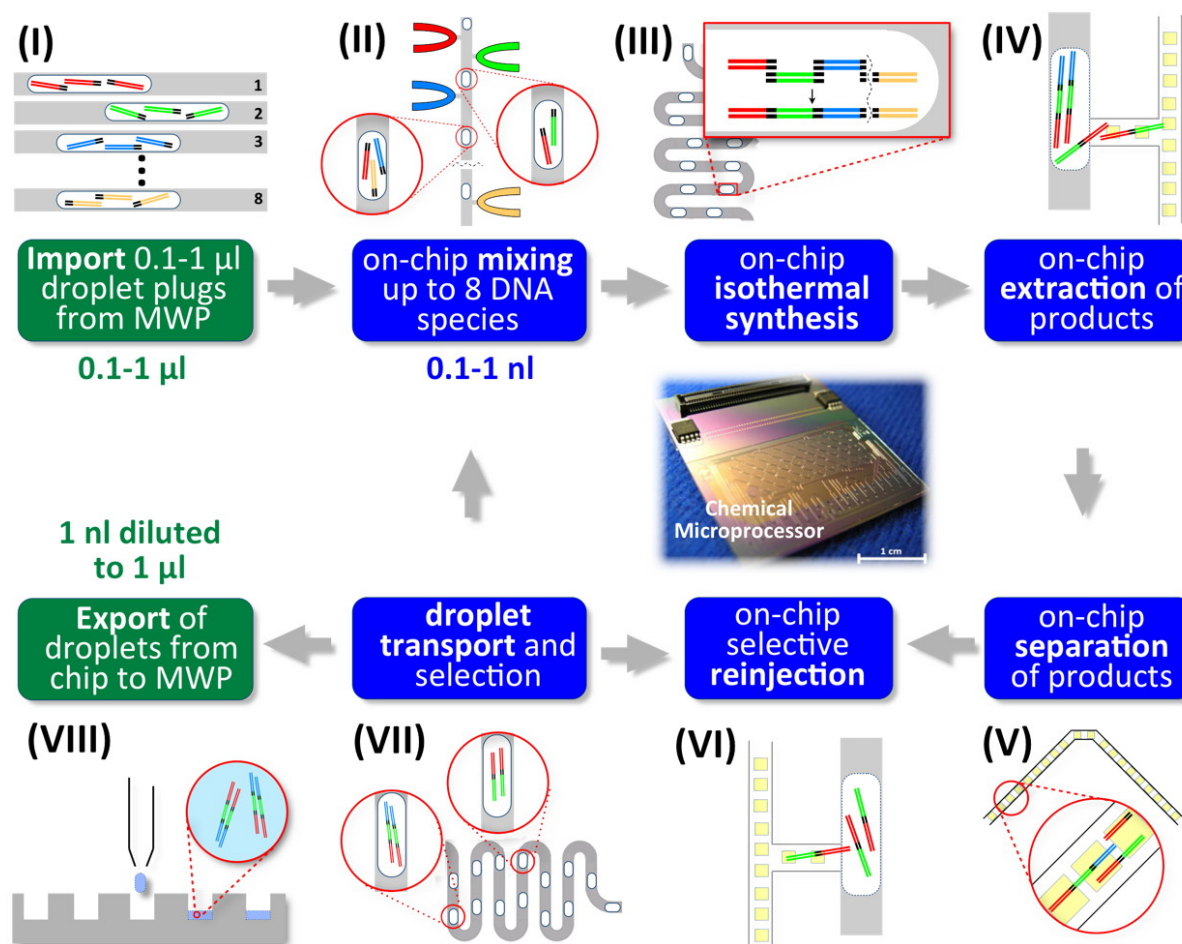
**Fig. 1 Fabrication flow:** (A) SiO<sub>2</sub> deposition 650 nm, (B) SiO<sub>2</sub> etching, (C) Ti/Pt (15 nm)/Au (200 nm)/Ti (10 nm) metal layer deposition, lift-off, SiO<sub>2</sub> deposition 200 nm, (D) RIE etching SiO<sub>2</sub>, (E) Photoresist deposition, (F) Ti/Pt (15 nm)/Au (200 nm)/Ti (10 nm) metal layer deposition, lift-off (G) 2nd SiO<sub>2</sub> passivation layer (400 nm) and RIE etching at electrode positions (H) ICP etching of 625μm Si for fluidic interconnection, sawing, resist removal, cleaning (K) O<sub>2</sub>-plasma bonding of electronic and microfluidic layer, FPGA/connector packaging by reflow soldering, fabrication of fluidic interconnection, tubing.

The fabrication process for a two-layer design is shown in figure 1. The procedure starts with the deposition and etching of an insulating thin-film layer (SiO<sub>2</sub>) after which a lithographic step is used to structure the first conducting

layer (Ti/Pt/Au), containing electrodes of the one-layer design as well as wires and vias for the upper (2nd) electrode layer (A-C). The following steps (D-F) include a second Ti/Pt/Au metallization process and an etching procedure to realize the upper electrodes and wires and to complete the via-connections. After opening the 2nd passivation layer at the electrode and conducting pad areas (G), fluidic I/O holes were placed using deep etching of the silicon substrate by Inductively Coupled Plasma (ICP) to allow a reverse side fluidic connection (H). The final steps are soft-lithography fabrication of multilevel PDMS micromoulds, which consist of an array of microfluidic channels and chambers, bonding the components using oxygen plasma as well as packaging via reflow soldering of a 0.4 mm contact pitch board to a Flexible Printed Circuit (FPC) connector with 100 contacts and digital thermometer elements incl. a unique 64-bit serial identifier (K).

## 4.2 Chemical Microprocessors – design and implementation

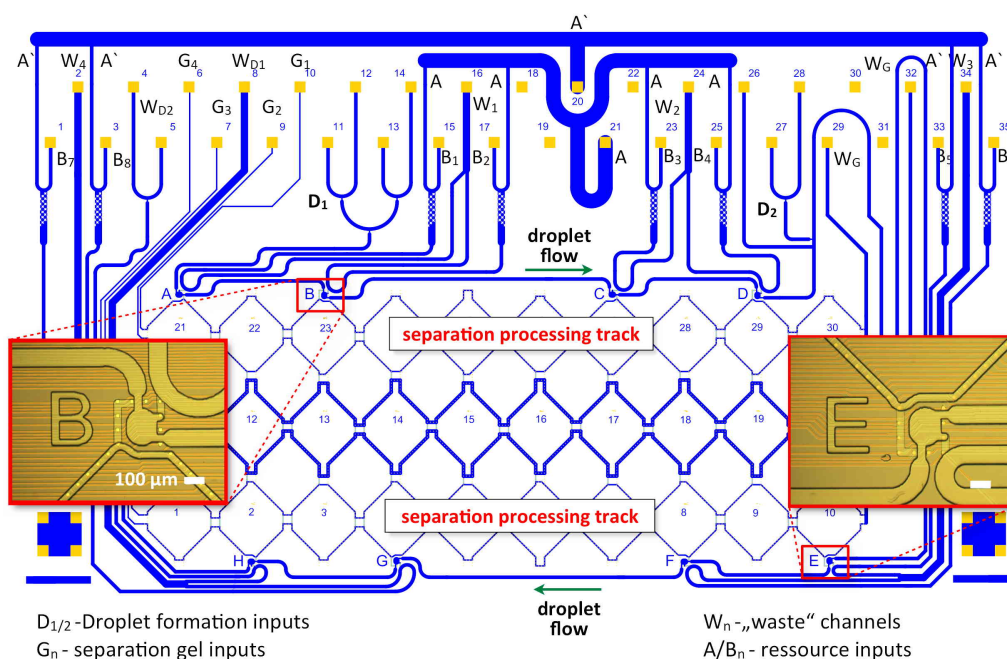
RUB designed and implemented electronic microfluidic processors (chemical microprocessors) to address isothermal DNA construction, in connection with isothermal amplification systems being developed at Weizmann and RUB (WP2). A DNA processor design was constructed that integrates droplet based I/O's with on chip reaction, electronic separation and sample transfer, with special structures suitable for multistage DNA assembly in CADMAD. The overall conception and implementation of the microfluidics architecture employed is illustrated in figure 2 and the integration of this concept to an overall DNA processor is shown in figure 3.



**Fig. 2 DNA processing steps:** Overview of specific processes of programmed DNA synthesis using microfluidics supported by microfluidics developed at RUB. For a detailed description of processing steps (I)-(VIII) see also section 3.1 of this deliverable. Light image close-up of chemical microprocessor chip\* completed in Year 1 of the CADMAD Project. (\* joint development ECCell project)



After designing and fabricating the first integrated droplet processor in year one<sup>7</sup> as shown in Fig. 3, the main focus in CADMAD was placed on (i) improved on-chip droplet handling including picodroplet generation, droplet braking and droplet-gel interfacing (ii) a separate combinatorial droplet generator chip with electronic composition control (iii) an enhanced voltage separation module for programmable on-chip DNA separation. The complex DNA processor integrates droplet based processing with on chip reaction, electronic separation and sample transfer for multistage DNA assembly in CADMAD. The work on iterative design optimization for the various sub-processes involved in DNA processing for synthesis is documented systematically in the appendix, along with film material demonstrating the functionalities of the chips.



**Fig. 3 Overall fluidic design of DNA processor** with 35 I/O connector (top of image). Eight different reactors (A-H) can operate with continuous resource flow and electronically regulated I/O to the gel separation network (centre). Multiple rounds of separation and reaction are possible with this rather general purpose device. The central channel region can be used for droplet pickup and output, or filled with gel for more extensive separation protocols. The design includes on chip droplet generators (e.g. inputs 11-12) and mixers (e.g. inputs 17-18).

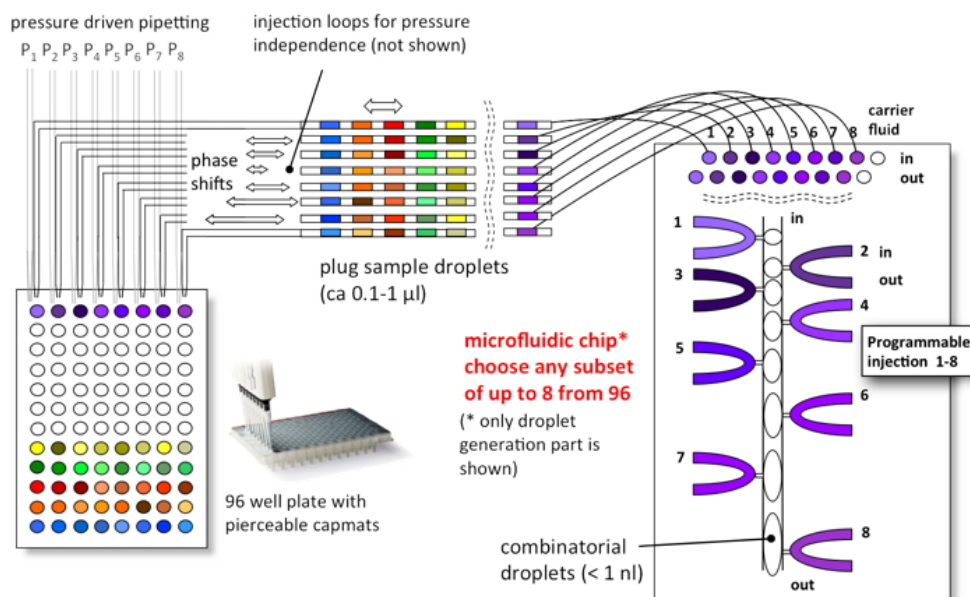
### 4.3 Main DNA processing operations on chip

In this section we detail the work towards realization of the separate functions of the DNA processor, following the 8 steps outlined above for the production of DNA libraries.

#### 4.3.1 Import of droplets from MWP - combinatorial interface (Step I)

While this was not the main target of this deliverable, which focuses on on-chip microfluidic processing, it provides a key component for interfacing these chips to standard I/O for connection to the rest of the project, and is included here for completeness. Substantial work in year 2 at RUB involved the realization of an electrowetting intermediate droplet processor to strengthen this interface, following the recommendations of the project coordinator.

<sup>7</sup> “In synergy with two other EU projects (ECCell, MATCHIT), a DNA processor design was constructed that integrates droplet based IO with on chip reaction, electronic separation and sample transfer, with special structures suitable for multistage DNA assembly in CADMAD.” Quoted from year one activity report.

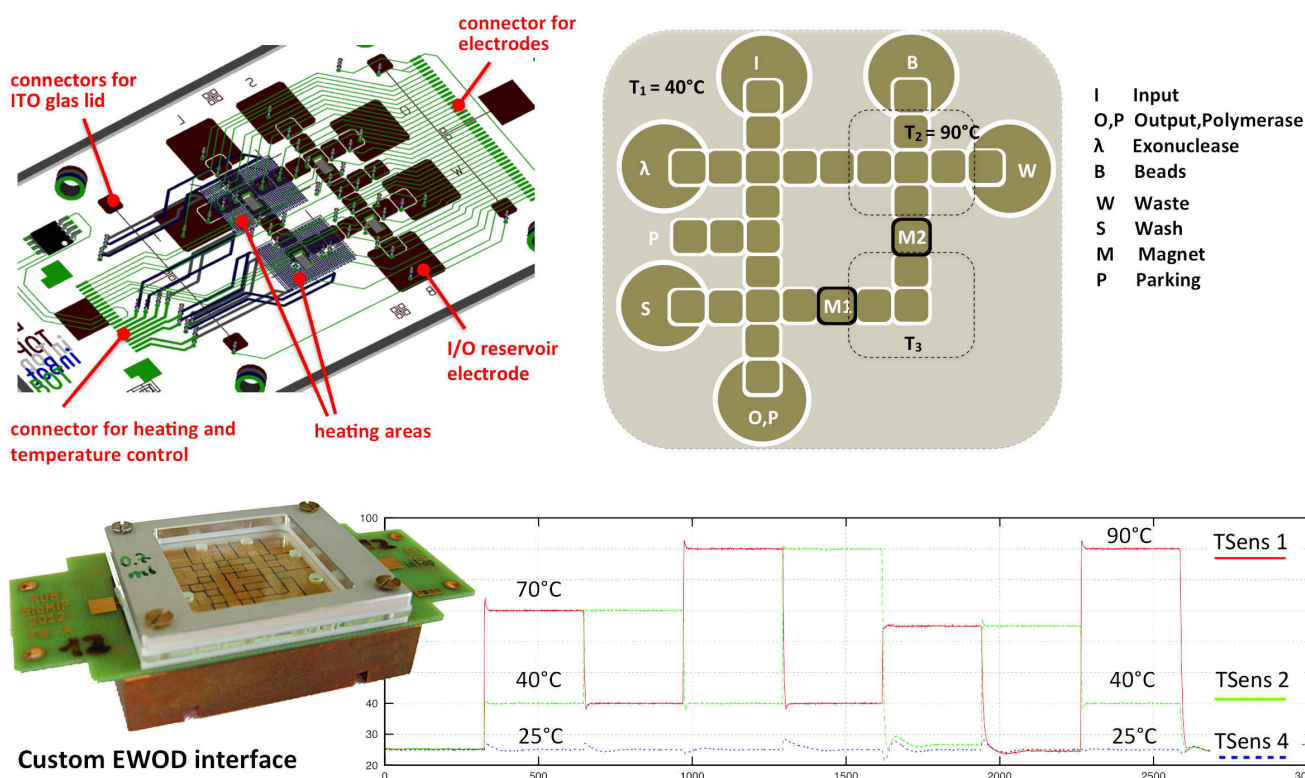


**Fig. 4 Schematic combinatorial interface microfluidics to MWP.**

A pipetting robot with up to 8 needles assembles 0.1-1 µl samples from a MWP as carrier fluid separated plugs in narrow bore capillaries (left). The relative phases of these 8 droplet chains is adjusted via pressure driven insertion loop control using valves. Sets of 8 such µl scale plug droplets arrive at microfluidic chip (right), where  $2^8 = 256$  binary (yes/no) mixtures can be programmed by the 8 injection ports to make ca 1 nl droplets (see Step 2).

In year 3 of the CADMAD project we will return to address the import of droplets from a multiwell plate (MWP) to the microfluidic chip. Especially, the novel on chip combinatorial droplet generation process developed in year two at RUB will play a central role in this processing. A direct link to microwell plates (MWP) using injection sampling into 8 chip channels as input and serial spotting as output will be set up by RUB. Figure 4 shows the concept of combinatorial interface microfluidics to a 96/384 well MWP.

In year two of the project, an alternative interface using a custom electrowetting on dielectric (EWOD) µl droplet processor (figure 5) was developed at RUB, to match other project interests in the consortium. Following a presentation of the Tecan EWOD devices, Ehud Shapiro and Tuval Ben Yehzkel (WEIZ), Marc Feiglin (Tecan) and John McCaskill (RUB), examined how the Y operation could be integrated on EWOD cartridges. The aim was to perform the Y operation at the sub µl scale in two successive rounds of PCR, ss-digestion and separation followed by ds completion of hybrid products. RUB then designed a custom electrode layout capable of implementing this scheme and built a test EWOD system according to this design as in Fig. 5. More information about this protocol for DNA processing as well as fabrication details, system integration (interface components, control software etc.) and proof-of-principle experiments are described in detail in the 2Y CADMAD WP3 report section 3.4.2. After ALL joined the consortium, taking over this milliscale integration, RUB's plan was to complete its custom EWOD development to allow a rapid interface to microfluidics to be set up, since ALL devices were locked into fixed interface structures associated with production. The final aim was to replace the valve circuitry, implementing the off chip components of figure 4, with an intermediate EWOD device, able to autonomously preprocess and select droplets (ca 0.3-1 µl) for input to the 8 channel interface to the microfluidic chip.



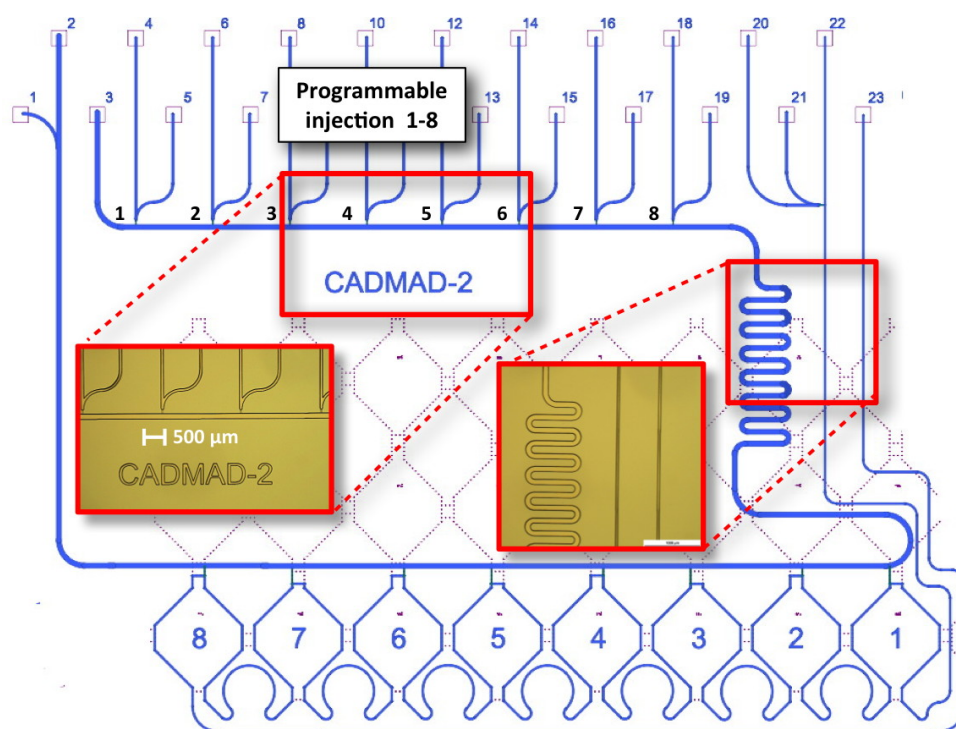
**Fig. 5 Alternative custom EWOD interface.** Upper left image: 3D CAD design. Upper right image: schematic electrode design with 36 independent controlled electrodes; position of the heating areas; example application of the Y operation. Lower left image: Complete manufactured EWOD PCB covered with a thin ITO glass slide and mounted on a copper heat sink. Lower diagram: On-chip temperature control with fast and programmable temperature ramps.

### 4.3.2 Droplet generation and combinatorial droplet mixing (Step II)

The key finding to allow defined droplets to be created was the invention of a back-pressure driven generation of plugs inside the microfluidic channels. The reason for the typical two lane input is to have a droplet resource channel which can also be used for cleaning the droplet-generation nozzle. Because of generating a plug inside the carrier-fluidic channel (typically with a water immiscible fluid as droplet carrier) this plug can be enlarged with additional material at later input locations and thus nearly arbitrary combinations of the inputs are possible. In Figure 6 a first microfluidic chip design to perform these combinatorial mixtures on-chip by 8 programmable injection ports as well as isothermal DNA-synthesis is shown.

The ability to stabilize liquid droplets with surfactants, allows droplets to be ported from one chip to another, and facilitated our development of separate chips for the DNA processor and combinatorial droplet generator.

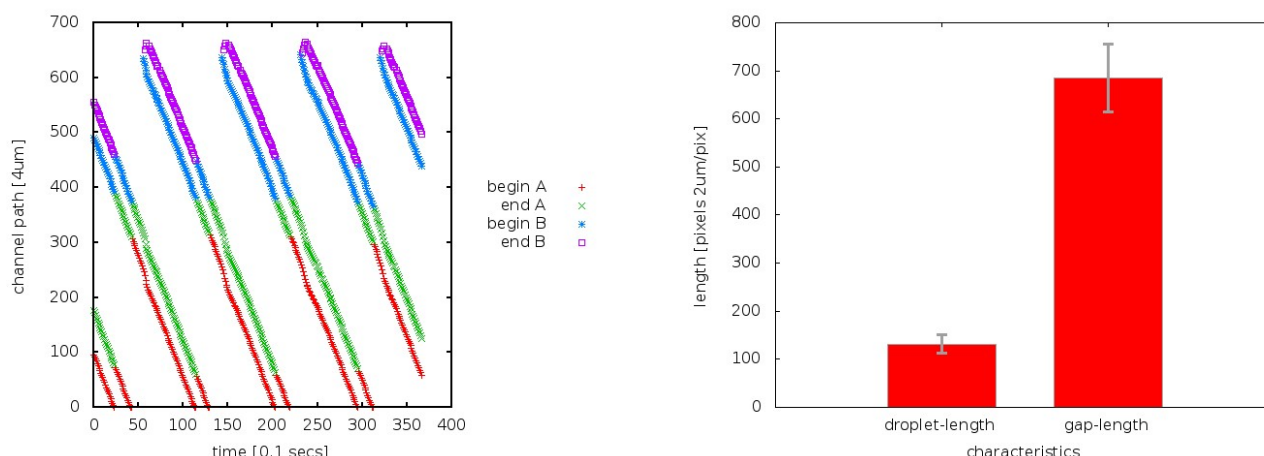




**Fig. 6 On chip combinatorial droplet generation:** CAD mask design (background) and first fabricated microfluidic structures (see two light microscopic images) to perform combinatorial mixtures on-chip by 8 programmable injection ports. The following channel's meandering shape allows prolonged reaction times of the on-chip isothermal DNA-synthesis. An additional feature of these CADMAD microfluidics are gel separation cycles (1 – 8) for separation of products. Between the eight separation loops and the main droplet flow-channel (thick line) shallow connections (typical height of 1-2  $\mu\text{m}$ ) are used to extract electrically material from the flowing by droplets. If required, this chain of droplets can be halted to give the extraction enough time to process. After extraction, with applying high voltage at distant electrodes these extracted charge molecules will be separated and then the looked for material will be selectively transported into a new wanted-for droplet.

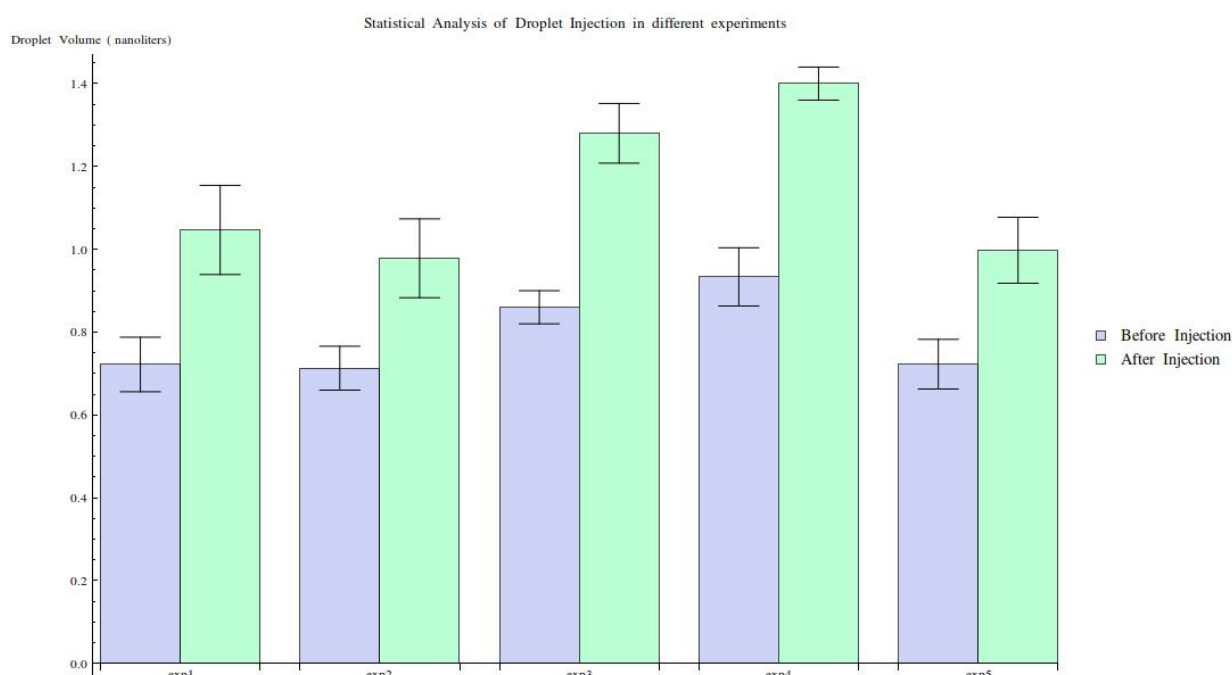
In former investigations we successfully tested several ionic liquids as a carrier fluid for on-chip droplet formation in microfluidics. We found out that butylmethylpyrrolidinium bis(trifluoromethylsulfonyl)imide [MeBu][NTF] as well as 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [EMIM][NTF] (IoLiTec Ionic Liquids Technologies GmbH, Germany) show a good compatibility with PDMS material (used for rapid prototyping of microchannels above the common microelectrode layer) and are successful candidates as carrier fluids regarding droplet formation performance. Several experimental systems were tested to include dye labeled oligomers into aqueous droplets formed in ionic liquids. Tests revealed that DNA does not penetrate the surface boundary into the ionic liquid. A disadvantage of the use of pure ionic liquids as separating fluid is that droplets may coalesce if they contact one another. This can be avoided by stabilizing the interface with surfactants. Therefore, more carrier fluids have been investigated in detail, including mineral oils and perfluorinated hydrocarbons (FC40) with appropriate surfactants.

Furthermore the wettability of the PDMS channels has been addressed by several means including silanization protocols. Recently most success has been achieved using a Teflon AF 1600 treatment (in FC-40: 0.2 % wt. for 10-15 min, drying, baking the chip for 30-40 min at 90°C) which also allowed us to use a broader range of carrier fluids and avoid swelling and absorption issues with PDMS. We obtained best results by using mineral oil with 4% Abil® EM4 as surfactant, see Fig. 7.



**Fig. 7 Extracted droplet features.** Shown are ten droplets (left picture, two of them viewable in the same frame, carrier fluid is mineral oil with 4% Abil) moving along a channel (length in this case 2.6mm). The droplets are 280µm long (1.4nl per droplet) and 1360µm apart. These droplets have been created with a defined length and a defined timing. The slight deviations, visible at around position 270 pixels, occur because of a 90 deg bend in the channel. 'Begin A' is either the head of a droplet or the beginning of a gap. The statistics on the right show the average length of around 170 droplets of 1.4nl created and the average distance in between these droplets of around 1.4mm.

An arbitrary mixture of droplet content is achieved by first creating a primary droplet with one ingredient and then adding successively further ingredients when the growing droplet passes by the next filling nozzle. There is no need to stop the moving droplet because injection is a rapid and controlled process. Mixing inside the droplets is also no serious issue because of the small volume (compared to the diffusion length) and high internal flow-dynamics resulting from the interaction of the droplet (plug) with walls in the channel of the carrier fluid. There is no need to merge independent droplets and as such no need for precise synchronization of two independent flowing droplets. To illustrate the feasibility of this concept some statistical tests are shown in Fig. 8.



**Fig. 8 On chip droplet injection/mixing statistics.** Primary droplets (purple) were created with a volume of ca 0.7 nl per droplet (violet box) and a secondary injection at a second nozzle was programmed with volume 0.3nl, resulting in elongated droplets (light green). The length is depicted in the boxes. The error bars reflect the standard deviation during the experiments (exp1: 12 droplets, exp2: 11 droplets, exp3: 3 droplets, exp4: 3 droplets, exp5: 8 droplets). Different combinations of timing and flow parameters were varied between the experiments.

Several experiments with differing parameters are shown to demonstrate the reliability of mixing substances in a single droplet. Because of the small droplet size, mixing inside the droplet is fast, and accelerated further by the

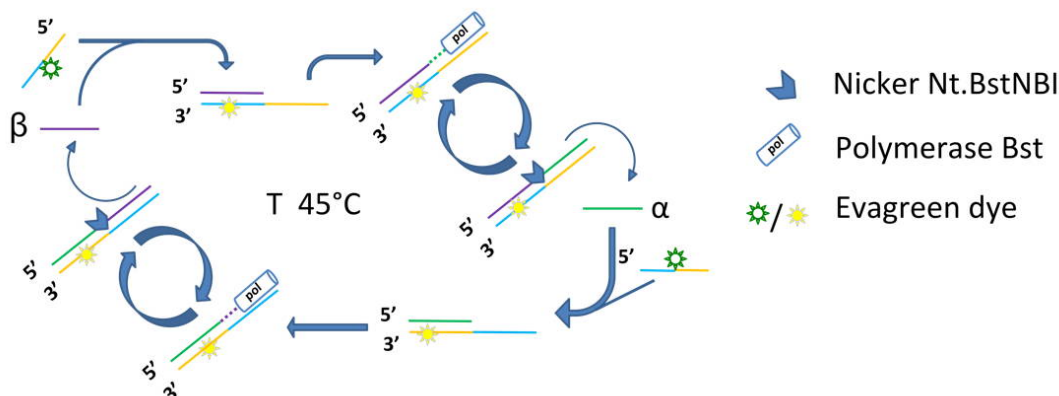
downstream meander (see Fig. 6). It must be emphasized that these droplets are being created when needed. This in contrast to other droplet-on-demand work<sup>5</sup> where the droplet parameters can be changed but droplets are always being created as strings of many droplets. A work which is closer to our approach is described in<sup>6</sup> where 4 air-pressure driven valves are required to create single droplets on demand which is at least two times the effort needed as for our approach.

### 4.3.3 On-chip DNA synthesis (Step III)

#### ***Isothermal DNA synthesis: short DNA pseudo-Y reaction***

The DNA synthesis test system we employed to test the microfluidic system is an isothermal (nicking strand replacement) reaction, with protected DNA templates, in the form proposed by Montagne et.al<sup>7</sup>. We first investigated this exponential strand displacement amplification on short DNA in the presence of nicking enzyme and DNA polymerase working in concert. We established the system in our lab and then (in Year 2) adapted it to run in a form close to the isothermal Y operation proposed by the Weizmann group, utilizing two separate templates to assemble mutually cross-catalytic templates: as shown in Fig. 9.

The reaction initially was studied in bulk on MWP (microwell plates, 10  $\mu$ l scale), the reaction outputs were analyzed in PAAG and the increase of the fluorescence signal of the intercalating dye was followed online in the MWP. When the enzymatic mixture with DNA as well as the dye was pumped in PDMS microchannels, we observed the intercalation of the dye inside the hydrophobic PDMS material during the reaction time (within 10 min), which made the detection system unreliable. It is known<sup>8</sup> that, the strong hydrophobic properties of PDMS and fast hydrophobic recovery after surface hydrophilization, negatively impacts on the performance of PDMS-based microfluidic device components. This problem does not affect dyes covalently bound to DNA.



**Fig. 9 Isothermal amplification test system - pseudo Y:** linking DNA strands  $\alpha$  to  $\beta$ . Based on a slightly modified Montagne system.

To decrease the undesired interaction with the PDMS walls, we modified channels to make them hydrophilic. The following method based on poly(ethylene glycol) (PEG) grafted PDMS channel walls was successfully tested and used in our microfluidic devices to switch the wettability of PDMS channels to hydrophilic behavior: The first step is a channel solution oxidation using  $H_2O/H_2O_2/HCl$  (5:1:1) to produce active silanol groups, but these are only stable for a couple of hours, because of the recovery of hydrophobicity. After special washing and drying steps we have treated the microfluidic PDMS channels with 2-[methoxy(polyethylenoxy)propyl]tri-methoxysilane using a slow continuous flow rate for 30 min.

After this treatment, and an additional step of washing the channel with 1 % BSA solution, the on-chip reaction at the 1nl scale was shown to synthesize DNA reproducibly with a sigmoidal amplification curve as shown in figure 10.

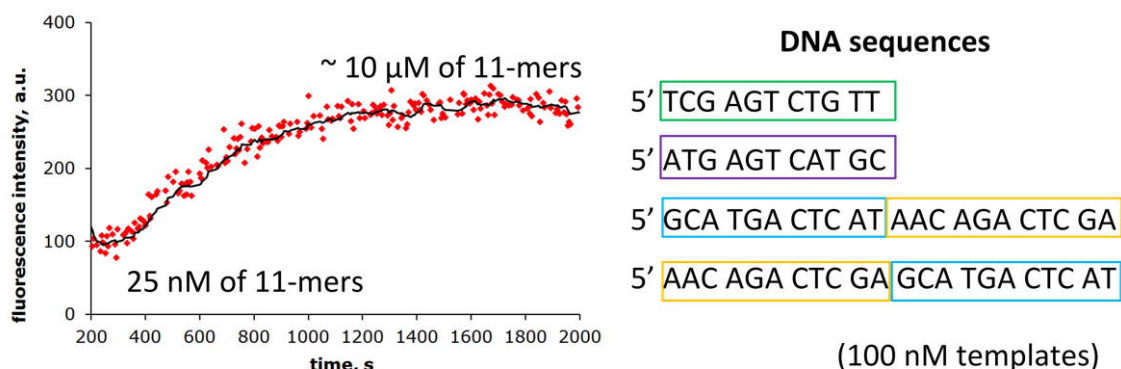


Fig. 10 Isothermal amplification in a microfluidic channel. Increasing TAMRA fluorescence shown.

### On-chip testing of isothermal amplification

The next step was to run the DNA synthesis reaction in droplets in the microfluidic chip. The channel treatment above negatively impacted the on-chip droplet formation because of the hydrophilic properties of the channel environment and therefore we decided to avoid it by substituting the intercalating dye used for detection. We had repeatedly observed that fluorophores that were covalently linked to DNA did not suffer from hydrophobic PDMS penetration.

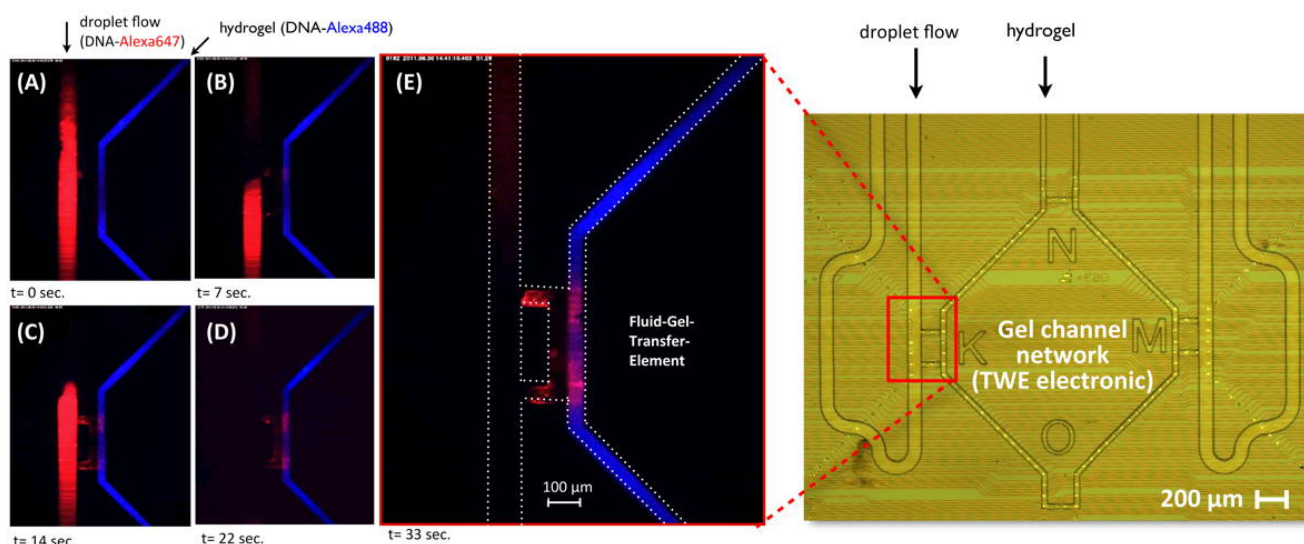
Rondelez et al.<sup>9</sup> presented a new scheme of Montagne exponential amplification with the detection based on N-quenching of 3'-terminal TAMRA fluorescent dye. We transferred the N-quenching detection scheme to our system with two templates (see figure 9). Thus we eliminated the problem of the PDMS walls interacting with the detection system and we were able to put the reaction into a droplet chain. Work on this reaction in droplets with various stabilizing additives is currently under way. The reaction in heavily stabilized droplets is inhibited, due to the micellar structures formed by the copolymer, as was confirmed by bulk measurements. We are currently investigating various additives and reaction conditions that will stabilize enzymatic DNA synthesis reactions in such small volumes.

#### 4.3.4 Product extraction from droplet into separation gel (Step IV)

To enable subsequent programmable on-chip DNA separation (step V), product extraction from droplets is required. We tested a simplified fluidic architecture with input and output droplet chains, in which isothermal DNA synthesis reactions can occur, as well as DNA extraction and separation in the central gelled region. We have demonstrated DNA extraction from droplet into separation gel. Each sample plug droplet containing fluorescently labeled DNA was separated from others by an immiscible carrier fluid. Figure 11 shows the electrokinetic extraction of sample material through the connecting shallow EOF (electro-osmotic-flow)-channel into the gelled channel. We used mixtures of triblock copolymers (Pluronic®) for the gel phase, which is fluid at low temperature and a gel above temperatures of about 25°C, depending on the composition of the gel.

For an improved transfer of material from droplet into the separation gel channel as well as the reinjection of separated products (section 4.3.6) into a new droplet it is useful to reduce the droplet flow by braking structures. Multiple iterations of droplet braking structures were performed in conjunction with simulation. The successful braking of droplets at sites for electronic processing is documented by video material in the D3.9 Annex.





**Fig. 11 Product extraction from droplet into separation gel.** The light microscopic image shows a modular part of the DNA Processor consisting of a gel separation network and two connected support channels for droplet flow. The left series of fluorescence images illustrate the transport of DNA (red) from a droplet to the separation gel (blue). (A) and (B) Droplets containing Alexa647 labeled 24nt DNA ( $10^{-7}$  M) were generated in the left support channel and passed the Fluid-Gel-Transfer-Element. The separation channel was filled with a mixture of 30% Pluronic (F87 : F127; 2:1) in His-buffer 50mM pH7.2 and Alexa488 labeled 12nt DNA ( $10^{-7}$  M) (C) - (E).

#### 4.3.5 On chip separation of DNA synthesis products by feedback wave electrophoresis (Step V)

A major task in the microfluidic realisation of the Y-operation is the separation of DNA-strands with the correct lengths from those with slightly different lengths. The idea we focused on was to use feedback to regularize the inhomogeneous response of local electrodes in Travelling Wave Electrophoresis (TWE). Usually, TWE requires analogue control potentials and works with a small number of individually controlled electrodes. Separation performance is dependent on maximally uniform fields, so that electrode placement is critical. The approach we investigated differs to enable the use of electrodes only at the bottom of channels may be used and of digital voltages. In compensation for this we have many more individually controllable electrodes. The regular phase relationships of the sinusoidal potentials in classical TWE were replaced by a continuous forward and backward tracking of the charged particles. The time constants used for forward tracking were chosen such that desired DNA was optimally drawn into the right direction and the unwanted DNA held back. As an unforeseen feature, it turned out that the fastest particle-type could be actively prevented from moving from left to right. This provides an absolute separation facility that is not known from classical electrophoresis.

#### Simulation

To achieve a connection between experimental separation and simulated feedback control, the RUB-control-software has been extended with an electrostatics based particle swarm package. The major design objective was to connect the control of an experiment in the microfluidic structure with a concurrent simulation. To realize such real-time simulation strong simplifications had to be made. A pseudo one-dimensional extension of the nonlinear PNP (Poisson-Nernst-Planck) equations enables rapid prediction of the local electric fields that are then used in conjunction with the real microfluidic designs to simulate up to around 1000 particles in the channels with real geometries. The simulation package is fully integrated into the RUB-control-software. The simulation itself is mapped via a camera-view into the user-interface or overlaid to the real microscopic image from the experiment and it thus represents an additional inlay of information to the normal experimental procedure.

Fig. 11 demonstrates that even in this difficult case a separation is possible though the yield is not perfect. The two slowest particle types are fully mixed and cannot be separated within only one channel. Three different types of particles are in the separating channel. The fastest particles with a relative mobility of 0.99 are shown as tiny bright crosses. The particles with mobility 1.00 and 1.01 are shown with grey circular shapes whereby the particles of mobility 1.00 are brighter than the slower particles. Though it cannot be seen clearly at this resolution, the fastest particles stay on the left half of the separating channel whereby the slower two particle-types move to the right.

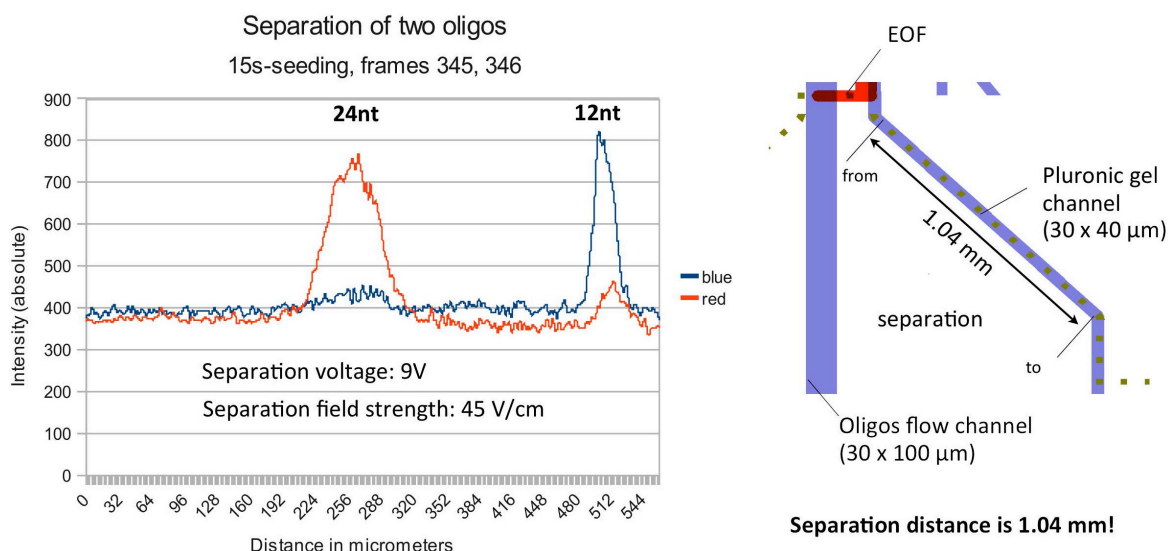


**Fig. 12 Simulated separation of DNA** with small (1%) mobility differences by feedback modulated local electrophoresis.

### Experiments

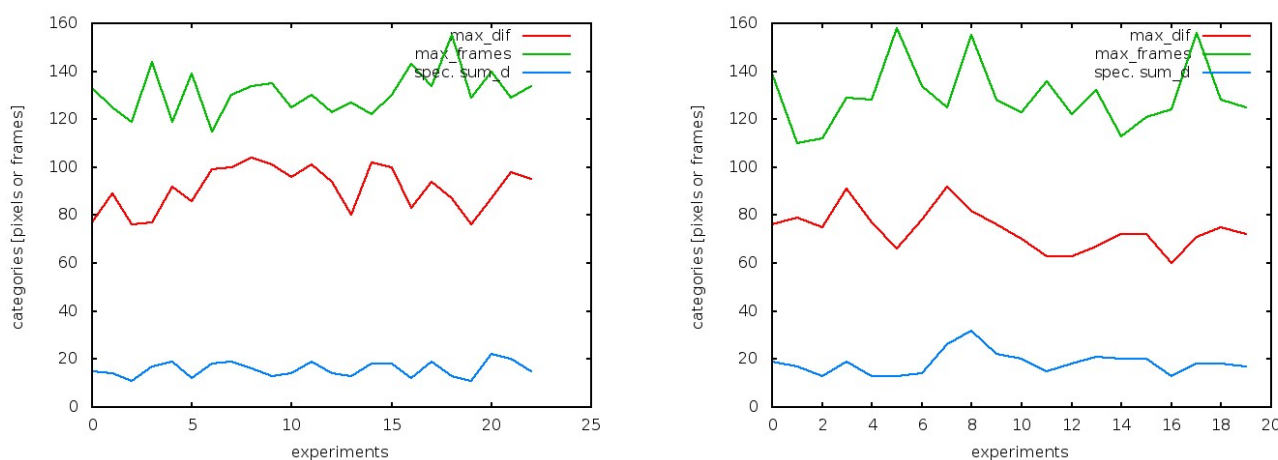
In simulations a 1% mobility difference could be separated but in experiments this technique of using embedded electrodes did not reveal a sufficient separation capability. Intensive research showed two major potential problems: firstly, the not optimal placement of the electrodes which, due to construction, had to reside on the silicon surface and secondly, unforeseen ionic cloud clusters which heavily perturb the separation and which could not be simulated in the above presented setup. Only with higher voltages (up to 10V) and electrodes far apart (around 1 – 2 mm) separation could be achieved reliably. Most experiments were done with short test oligos, which are more difficult to separate than longer sequences. An initial experiment with the original sequences delivered by Weizmann confirmed that separation will also be possible for double stranded oligos in the length range of 1000 nt, using similar reversible gel mixtures. The longer dsDNA behaved similarly to the short oligos and no signs are yet available that a separation will not be possible. Whether the accuracy can be trimmed to single nucleotides resolution is not yet known.

Successful on-chip separations in a Pluronics gel matrix are shown in Fig 13. Typical cases studies under a range of conditions involved two pairs of short oligomers (12nt and 24nt, 24nt and 45nt). The strands were separately labeled with red and blue fluorophores to facilitate analysis. After a separation time of around 6 min, continuing electrode actuation further leads to a fusion of the bands because of ionic depletion effects (right figure).



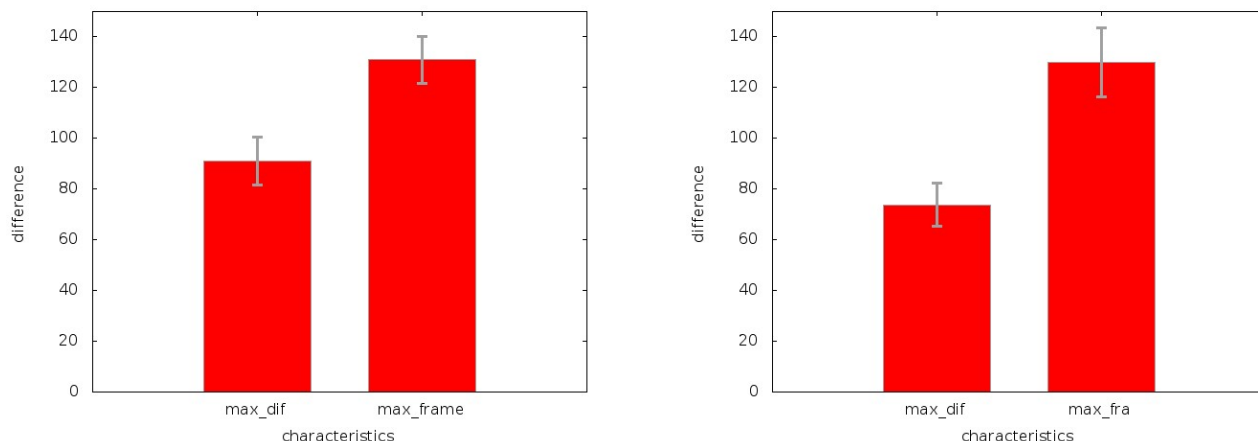
**Fig. 13 Product separation: Feedback wave electrophoresis.** Separation conditions: 50mM His pH7 + OligoN2 (24nt, Alexa647, red curve)  $1 \cdot 10^{-7}M$  + OligoFN2(12nt, Alexa488, blue curve)  $1 \cdot 10^{-7}M$ . Separation gel: 30% Pluronic 87:127 = 2:1.

In the following some systematic experiments are presented which should show the feasibility of separation in the microstructures, this time with sequences twice as long as in Fig. 13. A typical working setup were two electrodes (one with 0V and the other with +9V) sitting in the separation channel with a distance of 1 – 2 mm. The oligos to be separated (one 24-mer and the other 45-mer, and other short length combinations) were extracted from a DNA-containing solution (histidine buffer, 50mM, pH 7) via local electric fields into the Pluronic gel-phase and then separated by setting 0V potential at the entrance to the gel channel and +9V on the positive electrode at the other end of the separation channel.



**Fig 14: Separation experiments of mixtures of two DNA oligos.** Separation results were compared for ssDNA (24mer, 0.1  $\mu M$ , Alexa488 and 45mer, 0.1  $\mu M$ , Alexa647 nucleotides) on two different days and multiple runs in the same channel on a microfluidic chip. The gel used was Pluronic F87 30% w/v realized in a 50mM His-buffer at pH 7. The separating channel had width 40  $\mu m$  and height 30  $\mu m$ . The DNA concentration values were measured along the separation channel, see sketch on the right in Fig. 12. The plots record 3 values: the maximum difference in 2  $\mu m$  steps (max\_dif) between the two intensity peaks, cf. Fig. 13; the time when this maximum was observed (max\_f in seconds), the specific area between the two separating peaks throughout the separation (diff / frames).

In Fig. 14, the statistical reproducibility of separation experiments on separate days is presented. These experiments were done in the same microfluidic chip and structure. The maximum observed separation of the peaks in the DNA separation process is recorded for 20+ separation runs on each day, see Fig. 13 as a single trace example. The experiments were undertaken with differing electrical and timing parameters to find out the robustness of the separation. As seen from the figures, the experimental separation results are rather robust towards details of the separation process, especially considering that the curves are not corrected for the effects of the parameter settings.

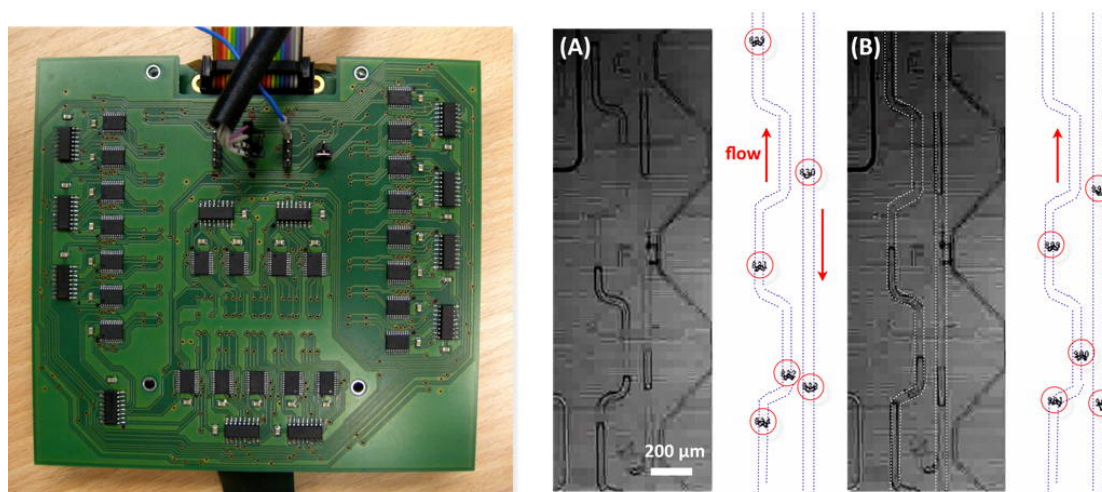


**Fig. 15: Statistical evaluation** of the same experiments presented in Fig. 12. Max\_dif represents the distance between the peaks of the two separated oligos in 2  $\mu\text{m}$  steps and max\_frame (or max:\_fra) the time (in seconds) when this maximum distance occurred.

In Fig. 15 the statistical evaluation of these experiments are shown. The error-bars reflect the statistical deviation of the measured values. The left plot comprises 23 separations and the right plot 20 separations. The time for extraction from the DNA resource channel varied between 10 and 60 seconds and the total separation time including extraction and replenishing the gel-channel for the next experiment differed by approx. 7 minutes.

### ***Improved electronic hardware interface and optical detection of droplets***

The on-chip purification and separation using travelling feedback electrophoresis developed in year 1 was further developed to resolve limits posed by non-linear space-charge depletion and other effects. In particular, as described in connection with the droplet recognition software, a new PCB board (Fig. 16, left image) was developed to allow larger analog voltages to be applied between more distant pairs of electrodes. This allowed the clarification of electrode end effects on the separation process and facilitated rather rapid local separation.



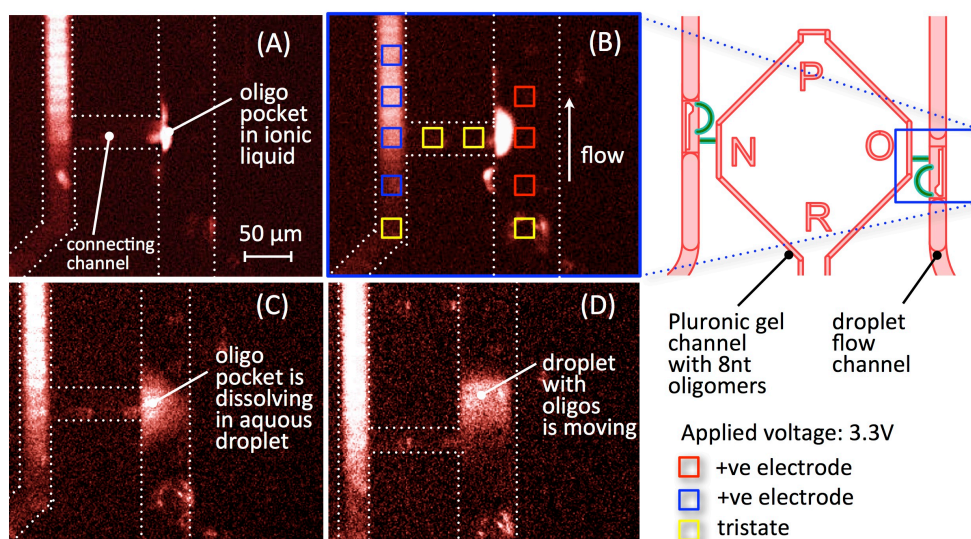
**Fig. 16 Improved separation and droplet detection.** Left image: New PCB board. Right: optical detection of droplet positions. The individual frames were extracted from a movie. The rightmost of the two snapshots is the situation at the chip a single timeframe later than the first image. The gray area is the corresponding camera image and in the white parts the results of image processing are displayed: the beginnings of the droplets are depicted with attached object identifiers.

The capability of the custom image processing for optical droplet detection is shown in Fig. 16. One can see the fronts of the droplets identified in the rightmost picture, tracking the droplets as they move in time.



#### 4.3.6 Product injection into droplet from hydrogel matrix (Step VI)

The next basic operation is the transfer of selected DNA content from the separation matrix to an output droplet at the nl scale. For this process, we set up a new microfluidic structure with two input and output droplet chains in opposite directions and a special device allowing temporary droplet parking at the extraction site (see Figure 17). DNA oligos are extracted (from a 30% Pluronic F87 gel) into a water droplet, separated by an immiscible carrier fluid. It turned out that it is better to create a dense extracted DNA oligo-pocket first at the boundary between the PDMS-wall and the ionic-liquid (figure 17, A+B) and then to wait until a water-droplet passes by taking the DNA with it (figure 17, C). Because of its electrical characteristics, the ionic-liquid acts like an electrode, asserting a high-field strength in the connecting shallow EOF (electro-osmotic-flow)-channel. When the water-droplet passes by, it takes up all the stored material at once (figure 17, D). This procedure has the advantage that a precise stopping of the droplet is not required, allowing subsequent operations without droplet parking structures, and thus the whole system remains stable while relaxing the control requirements.



**Fig. 17 DNA injection from hydrogel matrix into a droplet.** Pluronic concentrations and working temperature: 1. 22% Pluronic solution: 55°C 2. 30% Pluronic solution: 45-50 °C; DNA (in pluronic gel) conc. (8 nt, with Alexa-647) = 5  $\mu$ M, with 50mM His buffer. The carrier fluid is 1-Ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide. Shown is the injection of DNA oligos via the shallow connecting channel from the left gel-filled channel into a droplet at the injection site on the right. Because of the possible ionic exchange in the Ionic-liquid carrier of the droplet, a high field strength along the connecting channel is exerted and DNA is actively transported, building a pocket inside the Ionic liquid (B) where it remains until the next droplet passes (C). The water droplet merges with the content of the concentrated DNA in the pocket and diffusion mixes the DNA with the other content in the droplet (D).

#### 4.3.7 Droplet transport and selection processes on chip (Step VII)

Combinatorial droplets need to be selected out of the main flow, packed more densely and parked for reaction or other processing purposes, and switched back into the output flows for dilution. These processes are directed by external back pressure micropumps (Bartels) linked to various control capillaries in the device. The processes are monitored optically by white light microscopy. Ultimately the aim is to allow iterative droplet processing, as in the original overall design of Fig. 3 to facilitate multiple rounds of DNA processing.

In year 3, a new droplet design will be created which will allow droplets to be moving in a large circle, for an arbitrary number of rounds, like in a star-engine, with several combinatorial inputs at the segments of this circle to allow for subsequent injection and extraction of material from the droplets. The whole process will be observable online and detailed control of the droplet movement will enable arbitrary droplet processing algorithms.

#### 4.3.8 Export of droplets from chip to MWP (Step VIII)

The aim of this step (to be completed in year 3) is the export of nl droplets to individual wells of MWP.

This step is non-trivial because of the need to perform 1nl volume ejection (1 nl spotting) and the individual droplet control required. Export of the droplets from the microfluidic chip faces several challenges. The first challenge is the passage from the small cross-sectional area channels on the microfluidic chip (50x40µm) into the vertical fluid-connections of the silicon chip (Ø 300µm) and the Teflon-tube (Ø 200µm). This issue could be solved by an appropriate surfactant stabilization of the droplets, which prevented them from sticking in the passage or merging. A remaining issue to be solved in year 3 is the export of these nl or pl droplets into MWPs, which is more difficult if one would like to control the number and identity of the droplets being placed in the single wells, as in the case of DNA library synthesis with one DNA species per well. Usual spotting techniques must be validated to be compatible with these types of multi-phase fluids and optical detection of the spotted droplets might turn out to be indispensable. We plan to use specially coloured marker droplet sequences as binary addresses or identifiers to identify where we are in the droplet chain and control the choice of MWP well to put the droplets in.

## 5 Conclusions

The microfluidic design, construction, and functional testing at RUB in the first 2 years of CADMAD have established the core processes (steps 2-7) on the 1 nl scale required to engage with DNA editing in CADMAD. The IO steps 1 and 8 will be addressed in year 3 along with the scale up to longer sequences to enable DNA library generation.

After successfully building an electronic DNA processing chip in the first year of the project, the core functions of the chip were tested in the second year of the project and the design of the chip for each of the core functions improved. The basic DNA processing steps of reactive droplet generation, DNA extraction from droplets, DNA separation on-chip by feedback wave electrophoresis, and DNA injection into droplets were all demonstrated with the enhanced microfluidic chip. A separate combinatorial droplet generator chip was produced and combinatorial droplet generation demonstrated. The microfluidic designs employed are listed systematically in the Appendix and the film material completing a demonstration of functionality are also collected there.

A custom EWOD pre-processor was designed and constructed, which would avoid complex fluidic plumbing in the interface to MWPs, establishing a link to the millifluidic processing brought into the project by the new partner ALL. It is now intended, however, that the interface to MWPs will be completed with conventional nl dead volume valves. In future work, the overall function of the DNA synthesis chips will be extended to longer DNA in the complete Y editing operations using the Gibson reaction. This enables several synthesis steps to be performed at once, and thus shifts the burden of microfluidic integration from iterative to parallel processing in droplets. The plans in year 3 have been revised to accommodate this shift.

Note that because of downstream PCR amplification options, the quantity of DNA that must be produced is not a critical issue, so the microfluidic development does not aim at large scale-up of DNA quantities. The DNA products are to be exported from the microfluidic chip back to the EWOD PCB or MWPs for further processing. Linear isothermal amplification will be investigated in year 3 to bridge the gap between amounts of DNA required for sequencing (> 0.2 µg) and the <1 ng quantities in typical nl volumes. The main advantages of the microfluidic format are integration potential for multiple synthesis rounds and extreme parallelization of synthesis in very small volumes. Our design places the synthesis under full electronic control.

## 6 Abbreviations

List all abbreviations used in the document arranged alphabetically.

DNA (ss, ds)	Deoxyribose nucleic acid (single stranded, double stranded)
EOF	Electro-Osmotic-Flow
EWOD	Electrowetting-on-Dielectric
FPC	Flexible Printed Circuit
ICP	Inductively Coupled Plasma
MEMS	Micro-Electro-Mechanical-Systems
μl	microlitre (10 <sup>-6</sup> litre)
MWP	Micro Well Plate
nl	nanolitre (10 <sup>-9</sup> litre)
nSDA	Nicking strand displacement amplification
oligo	DNA oligomer
PCB	Printed Circuit Board
PCR	Polymerase chain reaction
PDMS	PolyDiMethyl Siloxane
pl	picolitre (10 <sup>-12</sup> litre)
TWE	Travelling Wave Electrophoresis

## 7 Revision notes & responses to queries

### 7.1 Answers to specific queries

- 1) Input concentrations of DNA employed were in the nM to  $\mu$ M range, depending on the experiment and system. For example for the nSDA amplification process concentrations of DNA template were typically <40 nM whereas for other reactions DNA concentrations were typically in the  $\mu$ M range.
- 2) Experimental verification was predominantly via on chip fluorescence imaging, as shown in examples. Labeling was predominantly by covalent end labeling of participating DNA using Alexa488 and Alexa647 dyes. Reactions were also conducted in bulk and analyzed by gel electrophoresis. The short lengths of DNA employed in most separation tests were single stranded DNA species. The same reversible gel matrices, variants of the triblock copolymer known as Pluronics, are known to be applicable to longer dsDNA species in the range of the CADMAD project. We collaborated with Weizmann to obtain longer dsDNA samples (delivered 1 Jan 2013) and initiated first experiments showing some separation with these longer dsDNA sequences (as reported in P2 PROGRESS REPORT, 3.3 WP 3 Automation of DNA processing: liquid robots & microfluidics; p. 14). Further work on these longer sequences was delayed by the need to resynthesize larger quantities of this DNA for systematic testing: a company that we engaged to do this synthesis failed with the primers delivered by Weizmann, after a several month delay. However, Weizmann is no longer convinced this is the right way to proceed, after their positive experience with magnetic bead separations on SPRI beads.
- 3) The DNA concentrations used for separations were in the range of 1  $\mu$ M, which is a similar range to that of products in both the nSDA and the Gibson reaction.
- 4) Tests of reproducibility and efficiency were performed, especially for the separation and droplet mixing processes.
- 5) A demonstration was not envisaged at the review and would have to be performed at RUB, because Weizmann does not have the custom microfluidic workstation lab environment necessary for such a demonstration. Furthermore, the whole process is not yet integrated: the deliverable is for the individual steps and these have not yet all been linked up into a single processing chain. It would have been fruitful however to bring copies of the microfluidic chips and the EWOD processor to the review to complement the pictures provided. Note that time for the video demonstration of RUB results in the presentation were severely curtailed by the extended video demonstration of millifluidic coloured droplet processing by ALL and Weizmann at the 0.3  $\mu$ l scale.

### 7.2 Responses to further review criticisms of D3.9

**1. "It does not appear clearly if the microfluidic chip will be really usable for the DNA processing steps required by the CADMAD project."**

As seen from the above context (steps I-VIII), the processes implemented in the microfluidic chips developed by RUB for CADMAD, do address key steps in the processing of DNA for DNA editing as envisaged in CADMAD. Technical problems at Weizmann with the realization of an isothermal amplification and Y operation have meant that we have only been able to test the synthesis processes with short DNA oligos (using the nicking SDA reaction). To resolve this problem in future, we are now investigating with Weizmann the suitability of a combination of the Gibson reaction with linear amplification off chip to complete the linkup with DNA editing of longer sequences.

**2. "The drafting is not rigorous enough to show the ability of the chip."**

We have endeavored to make the description of functionality more precise.

**3. "Chemical microprocessors are designed, fabricated and interfacing microfluidic chips via an EWOD preprocessor are proposed, but the ability of the whole device for the processing operations is not clearly demonstrated."**



The deliverable concerned the separate processing steps, not their complete integration. In fact, at the request of the coordinator, the original rapid progress towards full integration was replaced by a more detailed focus on the elementary steps.

**4. “Section 4.3 presents a series of preliminary tests, technical improvements, existing problems, current investigations, and simulations, but it does not clearly demonstrate the device’s ability for processing DNA.”**

The referees opinion about what is expected here both differs from the grant agreement and our expectations, and we see it as biased by the comparative ease of progress (incidentally after over 50 man years at ALL) with commercial handling of solutions at the much larger 0.3  $\mu$ l scale, or for very special purpose applications as in the commercial Agilent system. With intensive effort at the request of the project, we have addressed the issues of handling DNA solutions for synthesis in a programmable fashion at the sub nanoliter scale. This is not important for the immediate production of libraries in the project, but important to demonstrate that the CADMAD approach will in future be scalable to much larger libraries with necessarily much smaller volumes.

**5. “Does the chip work for the DNA fragment lengths and concentrations required for the processing operations? Is there any problem with the carrier fluid? Is the problem of wettability successfully and finally solved”**

We have already included larger DNA sequences in lengths up to 1000 bps on the chip, and have chosen our separation media to be readily adaptable to these lengths. Different carrier fluids have been investigated in detail, including mineral oils, perfluorinated hydrocarbons (FC40) and ionic liquids. Enzyme denaturation at interfaces can be addressed with appropriate surfactants such as fluoroalcohol surfactants (e.g. perfluorodecan-1-ol)<sup>10</sup>, PFPE-PEG block-copolymer surfactants<sup>11</sup> or Abil® EM<sup>12</sup>, which are also necessary for droplet stabilization. More work is necessary to demonstrate separation and reinjection at longer lengths compatible with the other processes above: this was envisaged for year 3 (see also revised plan). Wettability has been addressed by several means including silanization protocols. Recently (in May 2013) most success has been achieved using a Teflon deposition system, which has also allowed us to use a broader range of carrier fluids and avoid swelling and absorption issues with PDMS.

**6. “Only images are shown, where we would expect exhaustive tests with a full screening of all required experimental conditions. Are results reproducible, robust? After two years, we expect deliverable 3.9 to be more than a generic proof of concept.”**

We concentrated on graphical representation to communicate the character of the processes that we have investigated. We have now included some more statistical analysis in our revised report, documenting our systematic experimentation on some of the key issues. There were hard problems to solve at this scale, and we have made significant progress in doing this, so that we deem the expectations expressed by this comment as not appropriate. One must distinguish typical specific application development in industry from a “generic” pushing back of the envelope in DNA processing by a research lab: in which part of the desired functionality lies in the general purpose programmability of the system. There were more than enough CADMAD specific problems that we have had to tackle to reach this point.

**7. “This deliverable must be rewritten in a concise and convincing way.”**

That is what this text now endeavors to do. Note that we had adopted an incremental approach, not repeating or condensing information available in the proposal or the first year reports, and recognize that this was not helpful to understand the context of what we were doing.

## 8 Appendix

Contained in a separate document. Not for public access.

## 9 References

- <sup>1</sup> Montagne, K., Plasson, R. , Sakai, Y., Fujii, T. and Rondelez, Y. Programming an in vitro DNA oscillator using a molecular networking strategy, *Molecular Systems Biology* 7:466-7 2011
- <sup>2</sup> Tangen, U., Wagler, P., Chemnitz, S., Goranovic, G., Maeke, T., McCaskill, J. "An Electronically Controlled Microfluidic Approach towards Artificial Cells." 2006 *Complexus* 3, 48-57.
- <sup>3</sup> Wagler, P. F., Tangen, U., Maeke, T., & McCaskill, J. S. (2012). Field programmable chemistry: integrated chemical and electronic processing of informational molecules towards electronic chemical cells. *Bio Systems*, 109(1), 2–17.
- <sup>4</sup> Choi, J.-W., Kang, D.-K., Park, H., deMello, A. J., & Chang, S.-I. (2012). High-Throughput Analysis of Protein–Protein Interactions in Picoliter-Volume Droplets Using Fluorescence Polarization. *Analytical Chemistry*, 84(8), 3849–3854.
- <sup>5</sup> Yong Zeng, Mimi Shin and Tanyu Wang Programmable active droplet generation enabled by integrated pneumatic micropumps, *Lab on a Chip* 13:267-273 2013; Ashleigh B. Theberge, Estelle Mayot, Abdeslam El Harrak, Felix Kleinschmidt, Wilhelm T. S. Huck and Andrew D. Griffiths Microfluidic platform for combinatorial synthesis in picolitre droplets, *Lab on a Chip* 12:1320 -- 1326 2012.; Hao Gu, Michel H. G. Duits and Frieder Mugele Droplets Formation and Merging in Two-Phase Flow Microfluidics, *Int. J. Mol. Sci.* 12:2572-2597 2011
- <sup>6</sup> Feng Guo, Kan Liu, Xing-Hu Ji, Hui-Jiang Ding, Meng Zhang, Qian Zeng, Wei Liu, Shi-Shang Guo, and Xing-Zhong Zhao Valve-based microfluidic device for droplet on-demand operation and static assay, *APPLIED PHYSICS LETTERS* 97:233701(1-3) 2010.
- <sup>7</sup> Montagne, K. et.al. *Mol. Syst. Biol.* 7, 1–7 (2011).
- <sup>8</sup> Lei Y, Liu Y, Wang W, Wu W and Li Z 2011 Studies on Parylene C-caulked PDMS (pcPDMS) for low permeability required microfluidics applications *Lab Chip* 2011, 11, 1385.
- <sup>9</sup> Rondelez et al Presentation on 18th International Conference on DNA Computing and Molecular Programming in Aarhus, Denmark, August 14-17 2012.
- <sup>10</sup> Chabert, M., Chabert, M., Dorfman, K. D., Dorfman, K. D., de Cremoux, P., de Cremoux, P., Roeraade, J., et al. (2006). Automated Microdroplet Platform for Sample Manipulation and Polymerase Chain Reaction. *Analytical Chemistry*, 78(22), 7722–7728.
- <sup>11</sup> Holtze, C., Rowat, A. C., Agresti, J. J., Hutchison, J. B., Angilè, F. E., Schmitz, C. H. J., et al. (2008). Biocompatible surfactants for water-in-fluorocarbon emulsions. *Lab On A Chip*, 8(10), 1632.
- <sup>12</sup> Choi, J.-W., Kang, D.-K., Park, H., deMello, A. J., & Chang, S.-I. (2012). High-Throughput Analysis of Protein–Protein Interactions in Picoliter-Volume Droplets Using Fluorescence Polarization. *Analytical Chemistry*, 84(8), 3849–3854.