Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary information for: Micro-droplet arrays for micro-compartmentalization using an air/water interface

Fabrication of the microfluidic device. The MDA is fabricated in the clean room from 500 µm thick, 4" pyrex glass wafers. The result is a glass slide coated with FDTS to create a hydrophobic background carrying and array of circular spots that is pure pyrex glass. The steps of the process are:

1. Create Chromium pattern to serve as dicing guide and processing side indication.
    a. Spin coat photoresist: The wafer is primed with hexamethyldisilazane (HMDS), and then a 1.5 µm thick AZ 5214E layer is spun on the wafer using an automated spin coater. 3 mL resist is dynamically dispensed (800 rpm), and then spun for 30 seconds at 4000 rpm (2000 rpm/s). This is followed by 60 s soft bake at 90 °C.
    b. Masked UV exposure: The resist is exposed to UV using an aligner with a chromium mask carrying the chromium pattern. It is exposed for 5 seconds at an intensity of 7 mW/cm^2.
    c. Image reversal: The wafer is baked at 110 °C for 120 seconds to create a reversal of the image, and then exposed in the aligner without using a mask for 20 seconds at 7 mW/cm^2.
    d. Development: The pattern is developed on an automatic puddle developer for 60 seconds using AZ 726 MIF (2.38% TMAH in water).
    e. Chromium deposition: Chromium is deposited on the wafer using e-beam evaporation. A 200 Å thick layer is deposited at 1 Å/s.
    f. Lift-off: The areas masked by the photoresist is cleaned by sonicating the wafer in remover 1165 (DOW®) for 10 minutes, followed by sonication in IPA for 5 minutes and lastly, rinse in DI water.
2. Create the hydrophobic/hydrophilic array.
    a. Spin coat photoresist: As step 1 a.
    b. Masked UV exposure: The resist is exposed to UV using an aligner with a chromium mask carrying the array pattern. It is exposed for 8.5 seconds at an intensity of 7 mW/cm^2.
    c. Development: As step 1 d.
    d. FDTS deposition: A molecular vapor deposition (MVD) system is used to coat the glass wafer with FDTS. The machine begins by treating the substrate with weak oxygen plasma for 5 minutes to clean the surface, and then the reaction chamber is filled with FDTS and then water vapour which is allowed to react for 15 minutes before being evacuated from the chamber.
    e. Lift-off: To reveal the hydrophilic spots, the resist is lifted off using the same recipe as in step 1 f.
3. Dicing: Lastly the wafer is cut into 26x76 mm glass slides using the chromium dicing guide. An automated saw with a diamond blade is used for this purpose.

For successful formation of droplets, it is critical that the FDTS layer is of a good quality. If there is indication of poor quality film, it can be enhanced by treating the wafers with oxygen plasma for 1-5 minutes after step 1 f, to ensure that no resist particles remain from the first round of lithography. It is also

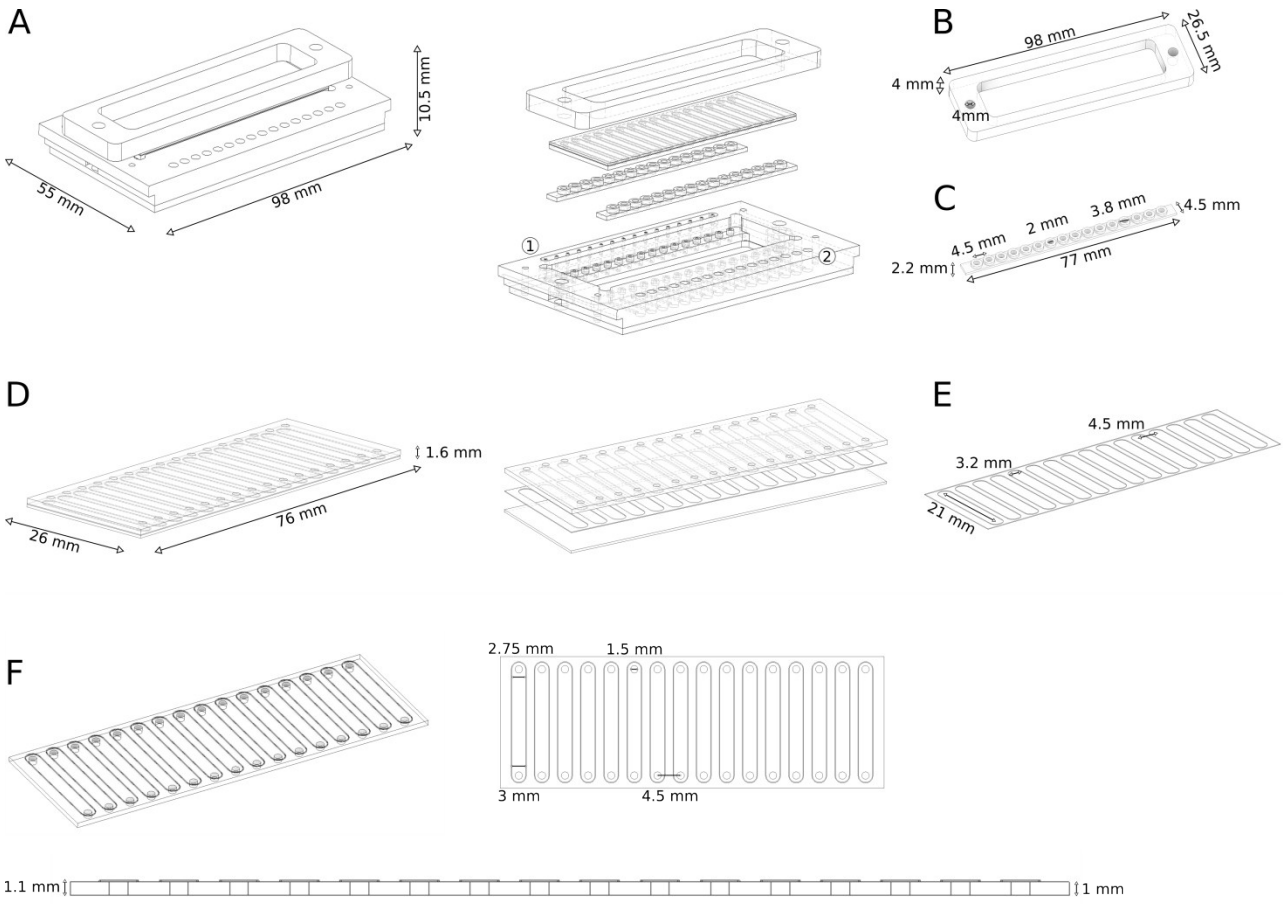
important to ensure that the development and lift-off processes are complete as left over resist on the glass surface will interfere with the ability to form droplets and to perform surface chemistry on the glass.

The array pattern consists of 200 by 2240 spots, each 5 µm in diameter with 5 µm spacing. For the larger droplets, 15 µm spots with 15 µm spacing, 30 µm spots with 20 µm spacing, and 50 µm spots with 40 µm spacing was produced on arrays 2x20 mm.

COC lid and chip assembly.

To form channels above the MDA, a lid is made by injection moulding, and inlets and outlets are drilled using a CNC machine. The lid is made from cyclic olefin copolymer (COC) and is bonded to the glass slides using tape bonding. The dimensions of the COC lid can be seen in the technical drawings in S.Fig 1, panel F, and an AutoCAD file is provided which contain the lid as well. The 100 µm tall protrusions on the lid keep the pressure sensitive adhesive tape (PSA) from coming into direct contact with the microfluidic channel, and ensures that the channel always has the same dimensions. The PSA film is 142 µm thick, but when subjected to pressure, it will squeeze out and fill the gap to the COC lid, with the final channel height being just 100 µm. The PSA is cut using a CO_2 laser system. The dimensions of the features in the PSA film can be seen in the technical drawing in S.Fig.1 panel E. The MDA glass slide, PSA and COC lid are placed together (see S.Fig 1 panel D) between two polished, flat aluminium plates and placed in a bonding press where the stack is subjected to a pressure between 1 and 2 kN for 1 minute at room temperature. It was observed that the adhesion was good and even, otherwise the chip could be rotated in the bonding press and subjected to another round of pressure until bonding looked even and complete.

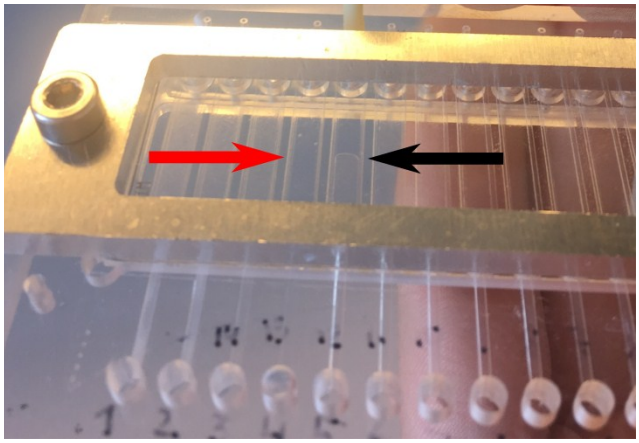
Fabrication of the flowsystem. To interface the chip, a reusable flow system was produced in PMMA by micromilling. It contains input reservoirs on the front side with channels leading to the inlet of the chip. This interface is sealed using PDMS gaskets (S.Fig 1 C). From the outlet, similar gaskets are used, and fluidic channels lead to the outlet pins on the flowsystem, which are tailored to fit into the tubing used on the peristaltic pump. The flowsystem is made up of two parts of PMMA in order to form the fluidic channels using micromilling. AutoCAD files of the two parts are supplied as supplementary material. To bond the two parts, they are first wiped with IPA, then, subjected to UV light for 120 seconds, before placing them together in a bonding press at 90 °C and 10 kN of force for 1 hour. After one hour, the heaters are shut off and the assembly is allowed to cool to around 50 °C before releasing the pressure. This creates a strong bond between the two PMMA parts. The PDMS gaskets are moulded in PMMA moulds which are fabricated using micromilling, and are press fit onto the flowsystem. The AutoCAD files for the mould are also supplied as supplementary material. The chip is held in place inside the flowsystem by a 4 mm thick aluminium brace (S. Fig 1 B), which ensures a tight seal against the PDMS gaskets. Nuts are inserted in the flowsystem to easily screw the brace in place using two bolts. AutoCAD files are supplied for all the milled parts and for the COC lid.



S.Fig 1 Technical drawings of the Flowsystem and chip. A) Assembled flowsystem with chip with final dimensions. The expanded view shows how everything is assembled. The reservoirs in the front (2) hold around 40 µL and can be connected to larger reservoirs for cleaning etc. On the back are connection pins (1) for tubing from the peristaltic pump. The chip rests on PDMS gaskets and is clamped down using an aluminum brace. B) The aluminum brace with dimensions. C) The PDMS gaskets with dimensions. D) The MDA chip with assembled dimensions. The expanded view shows how it is assembled. It consists of the MDA glass slide in the bottom, the pressure sensitive adhesive tape in the middle, and the COC lid on the top. E) The pressure sensitive adhesion tape with dimensions. F) The COC lid with dimensions. The tilted view shows the channels. The front view shows the dimensions of the structures, while the side view shows the height of the structures.

Droplet generation using oil/water

Droplet generation was done by displacement with fluorocarbon oil (HFE-7500 3M™ Novec™) to show that covering the array with oil was also feasible on this platform. The flow in the channels looked normal, and droplet generation was successful. We observed that a small amount of oil would stick to the outlet, but that was the only abnormality observed in the flow. Another observation we made was that the interface between the oil and water phase was nearly invisible, whereas the interface between water and air was clearly visible, as seen in S.Fig.2. This is less convenient in a research setting, as it makes is more difficult to determine if the channel is completely filled, and with which phase it is filled with, however, in an automated setting, this would be negligible. Besides these two observations, nothing else should make it impossible to use the oil phase as cover in cases where it is needed, and where diffusion through the oil is not posing an issue.



S.Fig 2 Photo of the interfaces between water and air (black arrow), and water and oil (red arrow) in the fluidic channels.

Digital ELISA for detection of ApoE3 and $A\beta_{1-42}$

Materials. Bovine serum albumin (BSA, Cat. No.: A7030-10G), N-Hydroxysuccinimide (NHS, Cat. No.: 130672-5G), N-(3-Dimethyl-aminopropyl)-N-ethyl-carbodiimide hydrochloride (EDC, Cat. No.: 03450-1G), Neutravidin (NAv, Cat. No.: 40945), Triton X-100 (Cat. No.: X100-100ML), and Ampliflu red™ (AFR, Cat. No.: 90101-5MG-F) were purchased from Sigma Aldrich. 3-Glycidyloxy-propyl-triethoxysilane was purchased from Evonik Industries (Cat. No.: Dynasylan GLYEO). Biotinylated poly-L-lysine-g-poly(ethylene glycol) (PLL-g-PEG-biotin) was purchased from Surface Solutions (Cat. No.: PLL(20)-g[3.5]-PEG(2)/PEG(3.4)-biotin50%). Heterobifunctional amine-poly(ethylene glycol)-carboxyl, HCl salt, average poly(ethylene glycol) molar weight 2,000 g/mol (NH_2-PEG_{2000}-COOH) was purchased from Jenkem Technology (Cat. No.: NH_2-PEG_{2000}-COOH). Monoclonal capture and detection antibodies against ApoE3 were kind gifts from Moravian Biotech (ApoE3-1.1 and ApoE3 3.1-HRP, respectively). The capture antibody was biotinylated and the detection antibody was conjugated to horseradish peroxidase. The monoclonal capture antibody for $A\beta_{1-42}$ was purchased from Synaptic Systems (Cat. No.: 295F2). The monoclonal detection antibody for $A\beta_{1-42}$ was purchased from IBL International (Cat. No.: 82E1) as part of the Amyloid-beta (1-40) High Sensitive ELISA kit. Synthetic $A\beta_{1-42}$ and human recombinant ApoE3 were purchased from rPeptide (Cat. No.: A-1166-1) and Sigma Aldrich (Cat. No.: SRP4696) respectively.

Buffers were prepared as follows. Sample Buffer was prepared as 0.05% (v/v) Triton X-100, 1.0 g/l BSA in 10 mM PBS. Rinsing Buffer was prepared as 10 g/l PEG_{20000} in 10 mM PBS. Labelling Buffer was prepared as 0.1% (v/v) Triton X-100, 10.0 g/l BSA, and 10 g/l PEG_{20000} in 10 mM PBS. Detection Buffer was prepared as 200 µM AFR, 1.0 mM H_2O_2, and 10 g/l PEG_{20000} in 10 mM PBS.

Covalent attachment of antibodies. Prior to surface functionalization, the MDA chips were cleaned by sonication sequentially in acetone, isopropanol and 96% aqueous ethanol solution for 10 minutes. MDA chips were submersed in a 1% (v/v) solution of 3-Glycidyloxy-propyl-triethoxysilane in 96% ethanol for 30 minutes then cured for 30 minutes at 110 °C. Next a poly-(ethylene glycol)-coating was applied by submerging the glass chips in a 100 g/l solution of NH_2-PEG_{2000}-COOH in 1.0 M $(NH_4)_2SO_4$ and 10 mM PBS for 2 hours at 40 °C. Slides were rinsed with ultra-pure water, dried under a nitrogen stream and stored in a vacuum desiccator. Next, the chips were assembled into a flow-system. To bind capture antibodies on the hydrophilic spots, NHS and EDC were dissolved in MES Buffer at a concentration of 50 mg/ml, mixed 1:1 to a final concentration of 25 mg/ml, and incubated in the flow-channel for 15 minutes. After a quick rinse of the flow-channel with MES buffer, a 0.1 mg/ml solution of monoclonal mouse antibody against $A\beta_{1-42}$ (prepared in MES Buffer with 0.05% (v/v) Triton X-100 added) was incubated in the flow-system for 30 minutes.

Finally, the flow-system was incubated with tris Buffer for 10 minutes to allow any excess of activated carboxylic acid-sites on the surface to react with the amine groups of the tris buffer. Subsequently, the antibody-functionalized MDA was used for digital ELISA detection of $A\beta_{1-42}$.

Attachment of biotinylated antibodies. Flow-system-integrated MDA chips were submersed in 1.0 g/l PLL-g-PEG-biotin in 10 mM HEPES buffer and incubated for 30 minutes at ambient temperature. Next, the chips were flushed with 10 mM HEPES buffer and incubated for 10 minutes with 0.05 g/l neutravidin in 10 mM PBS. After the incubation, the flow-system was rinsed with 10 mM PBS and then incubated for 20 minutes with 0.1 g/l biotinylated antibody ApoE3-1.1 (prepared in 10 mM PBS with 0.1 g/l BSA and 0.05% (v/v) Triton X-100 added). Excess antibody was removed by flushing the flow-system with 10 mM PBS for 10 minutes. The antibody-functionalized MDA was used for digital ELISA detection of ApoE3 immediately after the rinsing step.

Digital ELISA. Calibrator samples containing varying amounts of recombinant human ApoE3 or synthetic $A\beta_{1-42}$ as well as patient samples consisting of 10-fold diluted CSF were all prepared in Sample Buffer immediately prior to the detection assay. A 100-µl aliquot of the sample was slowly infused into the flow-channel at a nominal flowrate of 3.3 µl/min, which corresponded to a total incubation time of 30 minutes. Next, the flow-system was flushed with Rinsing Buffer followed by infusion of 100 pM detection antibody in Labelling Buffer. The antibodies were allowed to form surface-bound immunocomplexes for 30 minutes and then the flow-system was flushed with Rinsing Buffer for 10 minutes to remove excess detection antibodies. In the final step, a 5-µl liquid plug of Detection Buffer was passed across the flow-channel, thus leaving behind microdroplets loaded with detection reagents. Droplets hosting the detection antibodies produced strong fluorescence signals in less than 5 minutes after the droplet formation process was completed.

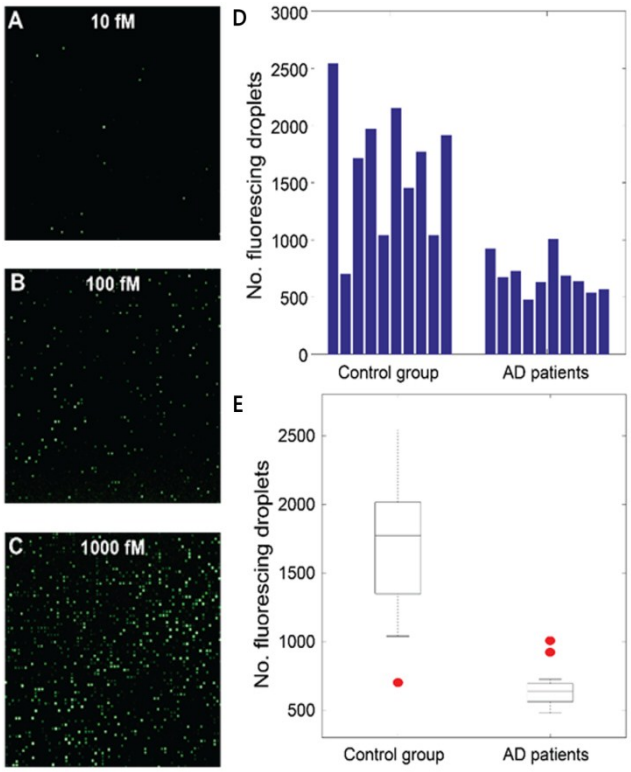
Clinical sample handling. Cerebrospinal fluid (CSF) samples from 20 individuals (9 male, 11 female, median age at sample collection: 66 years) taken in the period between 2007 and 2015 were analyzed. Samples were shipped on dry ice from Ulm, Germany, and upon arrival samples were stored at -21°C. The samples were divided into two groups; one group comprising samples from patients diagnosed with AD and a control group. The groups were tested in a blinded fashion and stratified after data-analysis. Prior to assaying, the samples were thawed on ice and diluted 1:10 in Sample Buffer. The control group comprised patients who obtained a lumbar puncture to exclude an acute or chronic inflammatory disease of the central nervous system. The group comprised the following diagnoses: tension headache (n=5), normal pressure hydrocephalus (n=3), exclusion of an inflammatory process (n=3), traumatic subarachnoidal bleeding (n=1), septic encephalopathy (n=1), migraine (n=1), myasthenia gravis (n=1), multiinfarct dementia (n=1), subclinical ischemia (n=1), depression (n=1), tardive dyskinesia (n=1), mutiple system atrophy (n=1).

Results

Digital ELISA detection of synthetic ApoE3 and $A\beta_{1-42}$. We applied our MDA platform to the digital detection of synthetic ApoE3 and $A\beta_{1-42}$. We adapted the MDA to conduct sELISA measurements by preparing two sets of devices in which the SiO_2-features were functionalized with capture antibodies against either ApoE3 or $A\beta_{1-42}$. A dilution series of synthetic $A\beta_{1-42}$ and ApoE3 was then incubated on the functionalized MDAs and subsequently detected by HRP conjugated antibodies. Representative fluorescence micrographs of the fluorescence signal generated from the digital ELISA of three concentrations of synthetic ApoE3 is shown in S.Fig. 3A-C. The ApoE3 antibody-pair exhibited a higher immuno-complexation efficiency compared to the $A\beta_{1-42}$-pair, as well as a lower non-specific binding of the detection antibody, and hence ApoE3-concentrations as low as 100 aM (2105 +/- 281 positive droplets) were still distinguishable from the background (1534 +/- 245 positive droplets). Nevertheless, despite the lower performance of the $A\beta_{1-42}$ antibody-pair, we still deemed the $A\beta_{1-42}$-device able to detect clinically relevant levels of the biomarker from patient samples.

Digital ELISA detection of $A\beta_{1-42}$ levels in patient CSF samples.

CSF samples from a control group, and patients clinically diagnosed with AD were analyzed using a MDA with 5 µm features in a sELISA assay as described above. $A\beta_{1-42}$ peptides from the CSF samples were captured by surface bound antibodies towards $A\beta_{1-42}$, and subsequently detected with an HRP-conjugated detection antibody against $A\beta_{1-42}$. A fluorogenic HRP substrate was employed to generate the fluorescence signal. The mean number of fluorescing droplets counted out of a total of 300,000 droplets were significantly lower for patients diagnosed with AD than in the control group (Mean (STD) were: AD group; 685 (164.7), and control group; 1629 (569.8), $p<0.001$ by Welch's t-test), see S.Fig. 1D. The groups were significantly different ($p<0.05$), however, there was considerable variance between the individual subjects and a small overlap between the groups (S.Fig. 3E). The variation in droplet count was mostly profound in the control group. Our findings obtained with the digital ELISA approach are in agreement with numerous published studies in which a decrease of $A\beta_{1-42}$ in CSF of AD patients was reported (for a recent review and meta-analysis, see e.g. [1]).



S.Fig 3 Single enzyme-linked immunosorbent assay detection of ApoE3 and $A\beta_{1\text{-}42}$. A-C) Representative fluorescence micrographs of MDAs functionalized to capture and detect ApoE3 at various levels of (A) 10 fM, (B) 100 fM and (C) 1000 fM. D-E) Digital ELISA detection of $A\beta_{1\text{-}42}$ in patient samples. D) Bar graph showing the level of $A\beta_{1\text{-}42}$ in CSF for 10 AD patients and 10 control patients. A total of 300.000 droplets were analyzed per sample. E) Box plot for the number of fluorescing droplets in the two patient groups. Red circles indicate outliers. The two groups were distinguishable despite inter-group variations ($p<0.001$).

1 B. Olsson, R. Lautner, U. Andreasson, A. Öhrfelt, E. Portelius, M. Bjerke, M. Hölttä, C. Rosén, C. Olsson, G. Strobel, E. Wu, K. Dakin, M. Petzold, K. Blennow and H. Zetterberg, *Lancet Neurol.*, 2016, **15**, 673–684.