Engineering solutions for flow control in microfluidic devices for spatial multi-dimensional liquid chromatography

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Abstract

Spatial multi-dimensional liquid chromatography can provide unprecedented resolving power by utilizing subsequent orthogonal separation mechanisms, while the analysis time is minimized thanks to parallel development of the analyzed fractions in each dimension. In this study, different engineering solutions have been realized aiming at achieving flow confinement and control during subsequent 1D and 2D chromatographic developments in a microfluidic device for spatial two-dimensional separations. First, the flow distributor design was optimized and physical barriers, i.e., reducing the cross section of microchannels as well as locally integrated monolithic substrates in chip segments, were assessed as a means of flow confinement. Furthermore, an on-chip active-valving, high-pressure approach was successfully developed. The flow in first-dimension separation is confined within a channel situated in a rotating axis containing through-holes. These are either closed during the 1st development or opened when aligned with the 2D flow distributor and the 2D channels after rotation of the axis by 90º, allowing for sample transfer and executing the subsequent 2D analysis. The on-chip active-valving concept has been successfully demonstrated in combination with stationary-phase analyte focusing prior to the 2D development using a microfluidic spatial 2D-LC device containing polymer monolithic stationary phases locally synthesized in the parallel 2D separation channels. Finally, this workflow was successfully employed to separate a mixture of dyes applying gradient RPLC during the 2D development.

Keywords: On-chip valves; Lab-on-a-chip; microfluidic devices
1. Introduction

Mass spectrometry (MS)-based proteomics has become an indispensable tool in biomarker discovery and validation studies [1–3]. It has been estimated that the human cellular proteome contains a cumulative copy number of $10^9$-$10^{11}$ protein molecules per cell [4]. To reduce the spectral complexity of contemporary life-science mixtures and at the same time prevent ion-suppression effects induced by co-elution, highly efficient separation methods are required prior to MS infusion [5]. The introduction of ultra-high-pressure liquid chromatography (UHPLC) combined with (long) columns packed with sub-2-micrometer particle technology has led to a significant gain in resolving power [6]. However, the peak capacity provided by one-dimensional UHPLC technology, even when utilizing long (shallow) solvent gradients, is not sufficient to account for hundreds of thousands of different protein isoforms [7–9]. Conventional column-based two-dimensional liquid chromatography (2D-LC) has the potential to reach very high peak capacities [10], when utilizing orthogonal developments [11], and its applicability in proteomics research has been readily demonstrated. A disadvantage is the sequential analysis of fractions sampled from the first-dimension development, leading to relatively long analysis times, making the technology intrinsically unsuitable for biomarker validation studies. Spatial three-dimensional liquid chromatography (3D-LC) constitutes a novel separation concept, in which analytes are separated by their position in a three-dimensional space characterized by ($X,Y,Z$) coordinates [12]. By utilizing orthogonal developments, very high resolving power can be reached since the maximum peak capacity is the product of the three individual dimensions [11,13]. In contrast to conventional multi-dimensional approaches where fractions are analyzed sequentially, spatial LC enables the develop of all fractions in parallel. This can result in unprecedented performance in
terms of peak-capacity-per-unit-time, \textit{i.e.} the peak production rate, allowing for biomarker discovery and validation studies.

The first prototype microfluidic device for spatial 3D-LC, integrating over 270 interconnected microchannels, was demonstrated in 2015 [12]. However, technology development for spatial multi-dimensional separations performed at high-pressure is still in its infancy. To generate a homogenous flow across parallel (2D) channels, flow distributors were developed based on prototypes for 1D-LC by Regnier \textit{et al.}[14] and Desmet \textit{et al} [15]. Based on insights obtained with computational fluid dynamics simulations and executing on-chip flow experiments, fractal bifurcating flow distributor designs were optimized [16,17]. Also, a fractal 3D flow distributor was developed to distribute a flow across 256 parallel channels filling the 3D space [18]. A critical aspect in establishing spatial separations is to restrict the subsequent developments within their predefined spatial domains, to effectively utilize all the available separation space [19,20]. A variety of different approaches, including pneumatic [21,22], electrokinetic [23–25], magnetic actuators [26], and ‘smart’ polymer materials [27,28], that can undergo phase changes have been explored to confine and block flows in microchannels. The current state-of-the-art microvalves are characterized by rapid response time times down to milliseconds. An alternative approach was presented by the Whitesides’ research group, who explored a torque actuated “TWIST” valve, where screws were used to collapse the microchannel fabricated in a poly(dimethylsiloxane) top substrate when pressed on the bottom glass substrate [29]. Guler \textit{et al.} developed a plug microvalve containing a cylindrical through-hole that can be aligned with microchannels via rotation, yielding a maximum pressure resistance of 2 kPa [30]. Unfortunately, many concepts are incompatible with the pressure resistance requirements of HPLC (5-40 MPa) or even higher. Furthermore, parallelization of the actuator operation to close and open parallel microchannels or a section of
the microfluidic device is an important aspect. Gong et al. reported a 3D printed multiplexer concept [31], integrating 5 parallel microvalves on-chip that can be actuated with a 15 ms phase interval and withstanding pressures up to 70 kPa. Recently, the use of freeze-thaw valves with switching times between 1 and 2.5 min was proposed by Nawada et al. allowing to withstand up to 20 MPa of pressure [32].

In this study, we explore different solutions to control and confine flow on a microdevice built for multi-dimensional spatial liquid chromatography. First the channel design was optimized, with respect to flow distribution across parallel second dimension (2D) channels. The performance of bifurcating flow distributors with a different channel layout was experimentally assessed, applying flow rates in a wide range from 50 to 1000 µL/min (at the distributor inlet). To confine the flow during the first-dimension (1D) development, the potential of using physical barriers was assessed. Microchannels with reduced channel cross section were integrated on-chip and the effects of implementing porous polymer-monolithic substrates to further enhance flow restriction were experimentally investigated. Finally, the development of an active-valve concept is presented providing a leak-free approach to confine developments at predetermined location.

2. Experimental

2.1. Reagents and materials

Butyl methacrylate (BMA, 99%), ethylene dimethacrylate (EDMA, 98%), 1-decanol (99%), cyclohexanol (99.9%), 2,2 dimethoxy-2-phenylacetophenone (DMPA, 99%) ethylene glycol diacrylate (EGDA, 90%), methyl methacrylate (MMA, 99%), benzophenone (BP, reagent plus, 99%), cyclohexane (anhydrous, 99.5%), sudan black B pigment, sudan I (dye content ≥ 95%), bovine serum albumin (BSA), fluorescein isothiocyanate (FITC), dimethylsulfoxide (DMSO),
sodium carbonate anhydrous (99.9%), sodium bicarbonate anhydrous (99.7%), and phosphate buffer saline (PBS) were acquired from Sigma-Aldrich (Zwijndrecht, The Netherlands). Monomers were purified by flushing the liquids through a column packed with activated basic alumina (VWR, Leuven, Belgium). 2-propanol (IPA, technical grade) was purchased from VWR. Dichloromethane (99.9%) and acetonitrile (ACN, HPLC supra gradient) were purchased from Biosolve (Valkenswaard, The Netherlands).

2.2. Chip fabrication

Fig. 1. depicts two designs of microfluidic devices for spatial 2D-LC, integrating physical barriers (Fig. 1A) and the optimized active-valve approach (Fig. 1B), respectively. The microdevices were constructed from cyclic olefin copolymer (COC) substrate (TOPAS advanced polymers, grade 8007, Kunststoff-Zentrum, Leipzig, Germany) using a computer-numerical-control micro-milling machine (Datron M7 Compact, Mühltal-Traisa, Germany). Prior to milling the microchannels, a face milling step was applied to reduce the surface roughness. The microchannels were constructed in both the top and bottom chip substrate by using a 200 μm diameter single-flut endmill and a 250 μm diameter ball-nose milling tool. For active valving (Fig. 1B). A 500 μm i.d. polyether ether ketone (PEEK) tube was threaded inside a perfluoroalkoxy alkane tube (PFA, Postnova Analytics, Landsberg am Lech, Germany), containing eight uniformly spaced and aligned 500 μm i.d. through-holes. In both chip designs, 400 μm i.d. through-holes were drilled in the bottom plate serving as 2D flow in and outlet. An irreversible bond between top and bottom chip substrates was obtained via solvent-vapor-assisted bonding [33]. After exposing the top substrate to cyclohexane vapor, bottom and top substrates were aligned with a precision of 2 μm.
using an in-house, custom-made, aluminum holder, and pressed together using a hydraulic press for 45 min.

Precision-machined bottom parts for a holder to accommodate the chip device were constructed from aluminum, featuring height-tunable side clamping blocks as well as flat-bottom nanoport connections (Upchurch Scientific, Oak Harbor, USA) compatible with 360 \( \mu \text{m} \) o.d. capillary fused-silica tubing. Top parts for the holder were milled from transparent PMMA and bolted together with controlled torque.

2.3. Integration of polymer-monolithic substrates

To enable a covalent attachment of the monolithic substrates to the COC channel wall, a surface pretreatment was performed. Therefore, microchannels filled with a mixture consisting of 50 wt% MMA, 50 wt% EGDA, and BP (20 wt% with respect to the total monomer content) were irradiated with UV light at \( \lambda = 254 \text{ nm} \) for 25 min (XL1000/F, Spectroline, USA), and flushed with ACN using an HPLC pump (LC Packings, Amsterdam, The Netherlands). Next, methacrylate-ester-based polymer monoliths were synthesized \textit{in-situ} in the microchannels applying a polymerization mixture consisting of 24 wt% BMA, 16 wt% EDMA, 54 wt% 1-decanol, 6 wt% cyclohexanol, and DMPA (3 wt% with respect to the total monomers). To restrict the polymerization at the desired location while applying UV light (\( \lambda = 365 \text{ nm} \) for 5 min), polyoxymethylene photomasks were constructed using micromilling. Afterwards, the channels were flushed with ACN.

2.4. Flow-control experiments with on-chip optical/fluorescence detection
BSA was labelled using 1 mg/mL FITC dissolved in anhydrous DMSO. A 2 mg/mL BSA dissolved in sodium carbonate buffer solution (0.1 M at pH = 9) was prepared. For every 1 mL of protein solution, 50 μL of FITC solution was added in small aliquots under continuous stirring and the reaction was incubated in the dark for 8 hours at 4°C. Next, ammonium chloride was added reaching a final concentration of 50 mM and the reaction was incubated for another 2 hours at 4°C. Excess of FITC was removed applying a NAP-25 gel filtration column and the protein adduct was recovered in PBS buffer. The on-chip monitoring of dyes and the FITC labelled protein flows was achieved with a digital optical/fluorescent microscope (Dyno-Lite, Naarden, The Netherlands).

Prior to sample loading, the monolithic stationary phase situated in the 2D channels was equilibrated by flushing several column volumes of a 95:5% (v/v) water:ACN and the 2D inlet and outlets were closed by means of an external 6 port-2 position valve. Next, fluorescent labelled protein dissolved in aqueous PBS buffer was introduced in the 1D inlet until reaching the channel outlet. In order to reassure a non-biased flow confinement test, the fluorescent tagged analyte was continuously injected until it reached the waste outlet of the system through the valve. After blocking off the 1D channel, the on-chip valving mechanism was rotated 90° aligning the through-holes with the 2D channels, and analyte focusing of a FITC labelled protein at the channel inlets was achieved applying 95:5% (v/v) water/ACN as the mobile phase at a flow rate of 100 μL/min.

3. Results and discussion

3.1. Chip design and advancing the channel layout

To realize multi-dimensional spatial LC separations, we have opted for a microfluidic chip design featuring parallel 2D channels. As the chip is composed of different substrate layers, the parallel channel design provides enough contact area to effectively bond the chip substrates,
yielding a device compatible with operating pressures over 10 MPa. As efficient packing of complex channel structures is extremely difficult to accomplish, implementation of monolithic substrates in microchannels starting from liquid precursors is more attractive. Since spanning of cross sections larger than 1 mm frequently resulted in rupture, we opted to integrate 500 µm i.d. 2D separation microchannels.

To effectively operate 500 µm i.d. (individual) separation channels containing a polymer-based monolithic stationary phase for gradient analysis of peptides or proteins, flow rates 10 up to 40 µL/min are recommended (depending on the macropore and globule diameter) [34], which corresponds to a flow rate at the 2D flow distributor inlet of 80 up to 320 µL/min, using the chip design with 8 parallel 2D channels, depicted in Fig. 1A. The performance of a flow distributor with a 175º angle between the bifurcations (previously reported optimal based on computational fluid dynamics modelling [16]) was assessed applying a broad flow rate range between 1000 µL/min down to 50 µL/min. Fig. 2A shows that the outer branches on each bifurcation level (marked with an asterisk) are less permeated than the respective inner channels, leading to an inhomogeneous flow profile. The inhomogeneity in flow distribution is more pronounced at lower flow rate, see Supplementary video 1 in the Supplementary Material (SM). When utilizing a flow distributor with a 180º angle between the bifurcations, a nearly perfect homogenous flow profile is achieved across all parallel channels, independent of the flow rate applied, see Supplementary video 2.

A spatial 2D-LC chip based on the design in Fig. 1A was developed, including the optimized bifurcating 2D flow distributor with three levels of bifurcations featuring an angle of 180º, a 500 µm i.d. circular channel for the 1D development, eight 500 µm i.d. parallel 2D channels (circular cross-section), and a flow collector. In an attempt to minimize leak flow during the 1D development into the 2D flow distributor and 2D channels, external valves were used, allowing to
introduce dye in the 1D channel meanwhile blocking off the in- and outlets of the 2D flow distributor and collector. In addition, the cross section of the flow distributor and the 2D channel inlets was reduced from 500 to 200 µm. During the 1D development significant leak flow was observed in the microchannels of the flow distributor and in the 2D separation channels, see Fig. 3A. To further decrease the permeability of the chip segments confining the flow during the 1D development, polymer-monolithic substrates were synthesized locally in the microchannels positioned vertically with respect to the 1D channel by utilizing photomasks, see Fig. S1. Fig. 3B and C show screenshots obtained during and directly after filling of the 1D channel, respectively.

Even at low pressure operation (0.7 MPa at the 1D inlet, open 1D outlet, and closed 2D in- and outlets) some leak flow was observed towards the 2D microchannels and flow distributor channels positioned closer to the 1D inlet, although to a much lesser extent compared to Fig. 3A. Fig. 3D shows sampling of the 1D effluent and elution in the parallel 2D channels. Note the highly homogenous flow profile in the 2D channels. However, in the current design, recovery problems (when sampling from the 1D channel to the 2D channels) are observed due to the limited number of 2D channels.

When considering spatial multi-dimensional separations, the first-dimension LC mode could also utilize a chromatographic support structure, thus the permeability differences between 1D and 2D will be less pronounced. This will inevitably lead to more enhanced leak flow, compromising the available separation space and, hence, minimizing the maximum achievable peak capacity. Based on this study, the bifurcated flow distributor with 180° angle and 500 µm parallel channel design were kept.

3.2. On-chip active valving
To advance flow confinement and prevent adsorption of analytes at the interface between the 1D and 2D development an on-chip active valving approach was pursued. This concept (see Fig. 1B) is based on a rotating axis, comprised of a PEEK tube (that constitutes the 1D separation channel), containing through-holes that either can be closed when these are positioned perpendicular with respect to the 2D plane or open when aligned with the 2D flow distributor and the 2D channels after rotation of the axis by 90°. Note that this prototype, in contrast to the device discussed in ‘Section 3.1’, does not incorporate physical barrier by reducing the cross-section diameter of the channels. A first demonstration of this concept was developed by applying solvent-assisted drilling to introduce a PEEK tube in a microfluidic device from poly(methyl)methacrylate, allowing to confine the 1D development. After drilling a small i.d. channel the PEEK tube was mounted in the drilling machine. Dichloromethane was sparsely added on the chip while the PEEK tube was drilled at low speeds into the existing channel, creating a tight fit between chip and tube. After evaporation of the dichloromethane a tight fit was obtained allowing to rotate the PEEK tube using a gripper, but not by hand, see Fig. S2 for a photograph of the prototype chip device. Although flow confinement was achieved, minor leakages from the 1D channel to the 2D channels appeared after repeated switches. A second-generation prototype was developed using COC as substrate and micromilling a support groove for the PEEK tube, see Fig. S3 [35]. The groove exactly matched the 500 µm i.d. × 1.58 mm o.d. PEEK tube. Furthermore, the chip substrate plates were bonded with the PEEK tube inserted ensuring alignment of the through-holes with the flow distributor and the 2D channels. Fig. S4A and B shows photo captures during the 1D development. Progressive filling of the tube is noticed by the appearance of black dye in subsequent through-holes perforating the PEEK tube. However, significant leakage was observed at low pressure
operation (0.15 MPa). Adamopoulou et al. recently reported on a similar concept and corresponding leakages at the channel inlet and interface [36].

To realize a leak-free device, the PEEK tube was inserted in a PFA sleeve (both containing through-holes), prior to solvent-vapor assisted bonding of the chip substrates, see Fig. 1B for the optimized chip design. A photograph of the device is depicted in Fig. 4A. The PFA sleeve is characterized by a high elasticity and can thus be compressed by the vertical forces applied via a custom-made encasing holder bolted using controlled torque. Successful operation of the active valving is demonstrated Fig. 5A-B and Supplementary videos 3 and 4, using dye and optical detection and FITC in combination with on-chip fluorescence detection. After confining the fluorescent marker solution in the 1D separation channel during the first-dimension development, the rotation of the axis is executed and FITC is effectively transferred by flow entering from the 2D flow distributor, feeding the parallel 2D channels while maintaining a relatively homogenous flow profile. No leak flow was observed during the first-dimension development applying pressures as high as 5 MPa, measured by using a restrictor at the 1D channel outlet, nor during the 2D development. Difference in flow velocity among the channels is likely induced due to elasticity of the PEEK tube during rotation while applying a high torque via the holder to prevent leakages, see Supplementary video 3. To univocally prove that flow was introduced in the chip via the flow distributor during the 2D development, a fluorescent dye tracer was added, invia an in-line short loop, in the mobile phase when recording Supplementary video 4.

3.3. Stationary-phase focusing and chromatographic separation

Stationary-phase focusing of analytes, due the strong interaction of analytes with the stationary phase when applying aqueous elution conditions (in RPLC mode), between subsequent
chromatographic developments (reducing the peak volumes) is highly desirable to counteract chromatographic dilution. When the tail of a peak experiences a mobile phase that is a stronger eluent (ACN-rich mobile phase), compared to the one at the front of the same band, it moves faster and a peak-focusing effect is observed. This methodology opens the possibility for scaling of channel dimensions such as to achieve high mass and volume loadability during the first-dimension separation using a large i.d. channel, and downscaling microchannels in subsequent 2D development to enhance the flow rate compatibility with for example mass-spectrometry detection, required for proteomics applications. Fig. 4 depicts the chip prototype and the zoom-in panels display a cross section of the microfluidic device highlighting the tight fit between the COC chip body and PFA sleeve as well as between PFA sleeve and PEEK tube, resulting in leak-free high-pressure operation. Applying a UV photo-initiated free-radical polymerization and a photomask, monolithic stationary phases were locally created in-situ, in the flow distributor and collector, in the through-holes of the PFA sleeve, and in the 2D separation channels displaying straight and well-defined interfaces, while no monolith is present in the PEEK tube that lacks UV transparency. The advantage of integrating the monolithic substrate in the flow distributor is that it reduces the gradient dwell volume. The first proof-of-concept of combining active-valving technology with stationary-phase focusing is demonstrated for a fluorescent labeled protein in Fig. 5C and D. BSA labelled with FITC dissolved in aqueous sodium carbonate buffer was introduced in the 1D channel and confined in the 1D channel via active valving, see Fig. 5A. After the 1D development, the axis was rotated and effective focusing of BSA was achieved at the 2D channel inlets applying aqueous elution conditions introduced via the 2D flow distributor at a flow rate of 100 µL/min, see Supplementary video 5. When introducing an ACN-rich (95%) mobile phase, the protein was remobilized while compressing the chromatographic band, and eluted towards the channel outlet,
see Fig. 5D. Differences in flow velocities and peak widths observed in the different 2D channels may be induced by inhomogeneity of the monolithic stationary phase or manual imprecisions when turning the rotating valve in the open position. Quantitative data and the corresponding discussion on channel-to-channel (n = 8) and chip-to-chip (n = 4) repeatability addressing mobile-phase velocity, band width, and intensity measured at the apex for fluorescent labelled albumin eluting during the 2D dimension development is provided in the Supplementary Material, see Figs. S4A-C.

The first demonstration of active valving for the flow confinement of a mixture of dyes (sudan black b and sudan I) during the 1D development, followed by stationary-phase focusing at the 2D channel inlets and subsequent chromatographic separation is displayed in Fig. 6. The mixture of dyes was dissolved first in ACN and then an aliquot of the mixture was diluted to a final composition of 30:70 % (v/v) ACN:H$_2$O. After active valving, a mobile phase composed of 5:95% (v/v) ACN:H$_2$O was introduced at a flow rate of 100 µL/min for 1 min via the 2D flow distributor allowing to focus the dye components in a narrow band at the inlet of the 2D channels filled with monolithic support structure, see Fig. 6A. Subsequently, a 10 min linear gradient reaching 90% ACN was applied, allowing to resolve the mixture components with baseline resolution. Fig. 6B presents a screenshot of the chip showing blue (more retained) and yellow bands, corresponding to sudan black B and sudan I respectively, eluting during the gradient. After travelling only 10 mm, baseline resolution was achieved between the dyes, as it can be noted in the extracted chromatograms from channel 6 (counted from the left) displayed in Fig. 6C. The data recorded via the optical microscope were converted and reconstructed in chromatograms using an in-house made MATLAB script). As expected, by increasing the travelled distance the resolution
is enhanced, see Fig 6D, as the incremental distance ($\Delta t_R$) increases more rapidly than the peak width.

4. Conclusion

The design of flow distributors of microfluidic devices for spatial multi-dimensional separations has been improved and different approaches for the realization of flow confinement were explored. Integrating physical barriers on-chip to confine flow during 1D, 2D (and possibly 3D) developments may work to some extent, but more likely compromises separation space and hence chromatographic performance. In contrast, an active-valving approach has been successfully developed to confine the flow during 1D developments in a spatial 2D-LC chip and applied in combination with stationary-phase focusing between the developments. The active valving approach allows to fully utilize the separation space provided by the chip, maximizing the potential peak capacity. To tackle the recovery problem, the number of 2D channels should be greatly increased. The stationary-phases focusing/remobilization concept is critical towards the operation of a spatial chip for spatial multi-dimensional LC since this counteracts chromatographic dilution. Furthermore, the stationary-phase focusing concept allows to tune the dimensions of the microfluidic channels in each dimension, i.e., having a relatively large i.d. 1D channel, maximizing the sample loadability and establishing small i.d. 2D channels to operate at low volumetric flow rates. This allows to hyphenate spatial multi-dimensional chip devices to mass-spectrometry imaging detection.

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References


**Figure 1.** Schematic representation of two different layouts of a microfluidic device for spatial 2D-LC. (A) shows a chip design utilizing physical barriers using cross-section constrictions (200 μm i.d. channels marked with red lines) in the flow distributor and the column head of the 2D channels. (B) a device equipped with a rotating active-valving system consisting of a PEEK tube threaded in a PFA sleeve, both containing 8 drilled through-holes. During the 1D development the through-holes are closed, during 2D development the through-holes are aligned with the 2D flow distributor and 2D channels. The black colored lines represent 500 μm i.d. channels and the green dashed line marks the effective separation space for the 1D channel.
Figure 2. Performance assessment of 2D flow distributors containing three levels of bifurcations and an angle between the branches of (A) 175° and (B) 180°, respectively. The inlet flow rate was 100 µL/min, resulting in a volumetric flow rate of 12.5 µL/min in each individual 2D channel with
a cross section of 500 µm. The red dotted line provides a guide for the eye highlighting the differences in flow velocities in the different 2D channels.

Figure 3. Screenshots of microfluidic devices for spatial 2D-LC utilizing physical barriers, i.e., the cross sections of microchannels situated at the 2D flow distributor and the 2D channel inlet are reduced from 500 µm to 200 µm i.d. (A) During filling of 1D channel (via bottom inlet) the 2D inlet and outlets are blocked off via external valves but significant leak flow is observed. In (B-D), monolithic support structures have also been synthesized in the 2D flow distributor and 2D channels, decreasing the permeability. (B) and (C) shows the 1D flow confinement during filling and only 20 s after filling the 1D channel with dye, respectively. (D) shows elution profiles of the 1D content transferred into the 2D channels applying at a flow rate of 100 µL/min at the 2D flow distributor inlet (5 seconds after start 2D development).
Figure 4. (A) Photograph of a spatial 2D-LC chip implemented with the active-valving concept and monolithic support structures at the flow distributor and 2D channels and (B-E) scanning electron microscopy images of cross and interface sections of the chip. (F) shows a zoom-in SEM image of the monolithic interconnected structure synthesized *in-situ* in the separation channel.
Figure 5. Leak-free operation of active-valving concept using on-chip fluorescent detection: (A) shows confinement after 1-D injection and development applying a flow rate 100 μL/min. (B-D) shows 2-D development: (B) is without stationary focusing yielding broad 2-D band, (C-D) is achieved with stationary-phase focusing of labelled protein captured at the 2-D channel inlet remobilization using a step gradient.

Figure 6. Demonstration of stationary-phase analyte focusing (A) and on-chip chromatographic separation applying gradient reversed-phase chromatography (B-D). Sample mixture was composed of sudan black B and sudan I. After 1-D flow confinement via active valving, focusing of the dyes was obtained by introducing 5:95% (v/v) ACN:H₂O at a flow rate of 100 μL/min for 1
min, followed by separation introducing linear aqueous-ACN gradient from 5% to 90% for 10 min via the 2D flow distributor. Chromatograms (C) and (D) were recorded at two different instances during the separation, closer to the 2D channel inlet (C) and outlet (D) respectively. Data were extracted from channel six counting from the left.
Supplementary Material for:

Engineering solutions for flow control in microfluidic devices for spatial multidimensional liquid chromatography

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1. Flow confinement via physical barriers

Fig. S1 depicts a photograph of a microfluidic chip for spatial 2D-LC with a single open \(\mu\)m i.d. 1D channel and a flow distributor and eight 2D channels. To confine the flow, cross section constrictions of 200 \(\mu\)m i.d. were incorporated and to further decrease the permeability of the chip segments for the 2D development, polymer-monolithic substrates were synthesized locally in the microchannels by utilizing photomasks.

![Photograph of a chip for spatial 2D-LC](image)

**Figure S1.** Photograph of a chip for spatial 2D-LC, based on the schematic depicted in Fig. 1A in the main manuscript that contains cross section constrictions and localized polymer monoliths in the 2D microchannels and flow distributor as means of flow confinement.

2. Active-valving prototypes

Fig. S2 shows a chip prototype integrating the active-valving concept developed in late 2014 by applying solvent-assisted drilling. In a first step a drill, with a slightly smaller diameter than the 3 mm o.d. PEEK capillary, was used to predrill a hole in the PMMA chip. Next, the PEEK tube was mounted in the drilling machine. Dichloromethane was sparsely added on the chip as the PEEK tube was drilled at low speeds into the existing channel, creating a tight fit between chip and tube. In this stage, as the PEEK is inserted in the chip, the 2D holes are drilled perpendicularly through the chip and PEEK tube. The perforated PEEK tube is then removed in order to allow drying of the chip, before it is reintroduced without solvent. Here, the
perforation (2D) holes are again aligned with those on the chip. Tight fits are obtained, where the PEEK tube can be rotated using a gripper, but not by hand. Water could be successfully pushed through the 1D channels, with the valve in closed position, without leakages to the 2D channels. However, with only minor back pressures, leakages from the 1D channel to the 2D channels appeared for the prototype. This emerges from the limited flow resistance between the 1D and 2D channels in close position: The perforating holes are 1mm in diameter, being quite large compared to the PEEK ID of approximately 2 mm, having a negative impact on the sealing performance.

Figure S2. Prototype of a microfluidic device containing a rotating PEEK tube as means of flow confinement.

Fig. S3 shows a second prototype of the chip fabricated via micromilling, implemented with an active-valving system consisting of a PEEK tube that contains 8 drilled through-holes. In the figure the valve is in closed position with the through-holes facing upwards, positioned against the COC plate that forms the main body of the device.
**Figure S3.** Second prototype of a microfluidic device containing a rotating PEEK tube as means of flow confinement. (A) Progressive filling of the channel is documented until the through-hole marked with the red asterisk. (B) Screenshot capture of the device 5 s after completely filling the 2D channels, demonstrating significant leak flow in the flow distributor and 2D channels.

### 3. Repeatability data

Fig. S4 provides a detailed insight in channel-to-channel, run-to-run, and chip-to-chip repeatability of the active valving system application and modulation of the 1D content in the 2D channels. Differences in peak width (A), signal intensity measured at the peak apex (B), and linear velocity (C) were recorded during the chromatography stages using the chip incorporating active valving, see the chip design in Fig. 1B in the main manuscript. Fig. S4A shows the peak width obtained during the focusing step, just before remobilization takes place, when applying a solvent step gradient. Note, the smaller the band width, the better the stationary-phase focusing is. Fig S4B shows the difference in fluorescence intensity measured at the peak apex. The different bars represent the performance measured for 4 different chips. The red error margins represent run-to-run standard deviation (SD) values for 3 consecutive measurements. Fig. S4C shows the repeatability in linear mobile-phase velocity measured at the moment of elution after applying a mobile phase step gradient. The error bars represent chip-to-chip SD for n =3.
**Figure S4.** Channel-to-channel, run-to-run (n = 3), and chip-to-chip (n = 4) repeatability data on peak width (A) and fluorescence intensity measured at the peak apex (B) during stationary phase focusing applying an aqueous mobile phase. (C) Presents the differences in linear mobile-phase velocity at the moment of elution after applying the solvent step gradient. The error bars represent the chip to chip standard deviation for 4 chips (A and B) and 3 chips (C), respectively.
4. Supporting Information videos

**Supplementary video 1.** Performance evaluation of a flow distributor with an angle of 175° between the bifurcations operating at a flow rate of 200 μL/min.

**Supplementary video 2.** Performance evaluation of a flow distributor with an angle of 180° between the bifurcations operating at a flow rate of 200 μL/min.

**Supplementary video 3.** Demonstration of active-valving concept introducing and confining dye during the 1D development, followed by active valving allowing to transfer dye towards the 2D channels utilizing flow fed via the 2D flow distributor.

**Supplementary video 4.** Flow confinement during the 1D development via active valving is demonstrated by the progressive lighting of the though holes with the characteristic fluorescent color, as well as successful transfer of the content in the 2D channels after rotating 90° the PEEK tube applying flow at the 2D flow distributor inlet.

**Supplementary video 5.** Stationary-phase focusing of FITC-labelled BSA protein at the inlet of the 2D micro-channels containing monolithic support structures applying a mobile phases of 5:95% (v/v) ACN:H₂O followed by subsequent remobilization of the proteins when applying a step gradient with mobile phase consisting of 5:95% (v/v) ACN:H₂O, at a flow rate of 100 μL/min.