Letter

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Interfacing Droplet Microfluidics with MALDI Mass Spectrometry: Label-Free Content Analysis of Single Droplets

Simon K. Küster,† Stephan R. Fagerer,† Pascal E. Verboket,† Klaus Eyer,† Konstantins Jefimovs,‡ Renato Zenobi,† and Petra S. Dittrich,†*

†Laboratory of Organic Chemistry, ETH Zürich, Wolfgang-Pauli-Strasse 10, 8093 Zürich, Switzerland

‡Laboratory for Electronics/Metrology/Reliability EMPA, Swiss Federal Laboratories for Material Science and Technology, Überlandstrasse 129, 8600 Dübendorf, Switzerland

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ABSTRACT: Droplet-based microfluidic systems have become a very powerful tool to miniaturize chemical and biological reactions. However, droplet content analysis remains challenging and relies almost exclusively on optical methods such as fluorescence spectroscopy. Hence, labeling of the analyte is typically required which impedes a more universal applicability of microdroplets. Here we present a novel interface coupling droplet microfluidics and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry for label-free content analysis of single droplets. Nanoliter aqueous droplets immersed in perfluorinated oil are created in a

microfluidic T-junction, transferred into a capillary and deposited on a high-density microarray MALDI plate mounted on a motorized *xy*-stage. The fully automated system is robust and reliable due to two unique features. Firstly, a simple optical droplet detection system is used to synchronize stage movement and exit of droplets from the capillary. Secondly, the microarray plate contains an array of over 26,000 hydrophilic spots within a hydrophobic coating, each spot acting as a recipient to confine the droplets and to prevent cross-contamination. The MALDI matrix can also be applied using our system by spotting matrix droplets on the microarray in a separate run. To demonstrate the potential of our system, we studied the enzymatic cleavage of angiotensin I by angiotensin converting enzyme and monitored the increasing concentration of the product angiotensin II over time. The interface provides a robust and fully automated method for rapid label-free and information-rich content analysis of single droplets. With the high number of droplets per plate, this method is particularly suitable for high-throughput screening applications.

Throughout the last decade, the generation and manipulation of ultrasmall-volume droplets on microfluidic platforms has opened fascinating opportunities for chemical and biological research.[1-5] Monodisperse droplets encapsulating femto- to nanoliter aqueous volumes can be generated at high throughput (up to kHz) within a hydrophobic carrier stream and can serve as individual microcontainers isolating biological or chemical species.

In recent years, many novel on-chip droplet manipulation techniques and interfaces have been developed. Droplet generators can be interfaced with liquid chromatography (LC)[6] or capillary electrophoresis (CE)[7-8] to alter the droplet content. Additionally, cartridge sampling techniques enable loading of droplets for reaction screening at high throughput.[9] Furthermore, a modular droplet toolbox is available enabling on-chip micromanipulations including ultrafast mixing

inside droplets,[10,11] droplet fusion,[12,13] splitting,[10,14,15] injection[16] and sorting.[17-19] These developments have enabled complex high-throughput experiments with strongly reduced reagent consumption making this concept highly attractive for various chemical and biological applications such as synthesis,[20] protein crystallization,[21,22] cell-free protein expression,[23] single-cell studies[24,25] and directed evolution.[19,26]

The analysis of droplet content, however, remains challenging and limits the applicability of droplet microfluidics. As droplets are commonly transported at high speed through closed microchannels, the lack of direct physical access renders in-situ measurements non-trivial. Therefore, the detection of the droplet content commonly relies on optical methods, mostly fluorescence microscopy. This significantly restricts the applicability of droplet microfluidics due to the required labeling of target molecules with fluorescent dyes.

Mass spectrometry (MS), on the other hand, provides label-free detection of a wide range of analytes and is therefore ideal for droplet content analysis. However, interfacing droplet-based microfluidic platforms (water droplets in oil) with MS instruments is challenging. Up to now, most of the effort has been directed towards developing on-line interfaces with electrospray ionization mass spectrometry (ESI-MS). However, the injection of alternating water and oil plugs interferes with the formation of a stable ESI plume. Attempts to overcome this problem have been made by using air as continuous phase[27] or by extracting the droplet into a continuous aqueous stream before creating the ESI plume.[28-30] The former approach renders more advanced droplet manipulation impossible while the latter approach suffers from Taylor dispersion of the droplet content after extraction which can result in cross- contamination. Additionally, the dilution of the droplet content impedes optimal detection. As an alternative, interfaces with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) uncouple the fluidic

system from the mass spectrometer and can therefore overcome these difficulties. MALDI-MS is particularly powerful in analyzing larger molecules such as peptides and also has a high salt tolerance. In many previous studies, MALDI-MS has been coupled to separation methods such as capillary electrophoresis or liquid chromatography by continuous deposition of the fluidic fractions on a movable MALDI target,[31-37] but up to now only few approaches have been presented to combine continuous droplet microfluidics with MALDI-MS.[38,39] Furthermore, the analysis in these studies was either not performed at the single-droplet level or manual spotting of individual droplets on standard MALDI-MS plates prevented high-throughput droplet chemical analysis.

Here we present a novel interface between droplet microfluidics and MALDI-MS that enables fast, precise and cross-contamination free deposition of up to 26,000 individual aqueous droplets on a micro-array plate for subsequent mass spectrometry analysis. The system is fully automated and therefore facilitates high-throughput analysis of droplet microreactors. We recorded mass spectra of compounds encapsulated in microdroplets with comparable sensitivity to MALDI-MS measurements on conventional stainless steel sample plates. Furthermore, we demonstrate the potential of the system by monitoring the formation of an octapeptide (angiotensin II) from a decapeptide (angiotensin I) during an enzymatic reaction over several hours.

A scheme of the interface is depicted in Figure 1a. Aqueous droplets of 3 nL were created in a microfluidic T-junction by injecting an aqueous phase into an immiscible oil phase (perfluorodecalin). The microchannel was connected to a perfluoroalkoxyalkane (PFA) capillary (360 μm O.D., 100 μm I.D.), the end of which deposited droplets on a micro-array plate mounted on an *xy*-stage (Figure 1b & c). The micro-array plate consisted of a standard stainless steel

MALDI-MS plate (123x81x1.2 mm^3), which was coated with a hydrophobic Teflon layer. Subsequently, the hydrophobic layer was structured by picosecond laser ablation[40] to form an array of 26,444 circular hydrophilic spots each of 300 μm diameter (Figure S-1). Every spot acted as a recipient for one individual microdroplet.

Additionally, an optical detection system consisting of a photodiode, a photodetector and an optical fiber (with a drilled hole) registered every droplet leaving the capillary (see SI). A real-time data evaluation system was used to differentiate aqueous droplets from oil plugs by performing a threshold analysis. When a droplet was detected, the stage was triggered after a predefined delay time to move the micro-array plate to the next empty hydrophilic spot. The movement was performed while an oil plug was exiting the capillary, therefore preventing splitting of droplets across multiple spots. Additionally, the detection system facilitated reliable deposition of microdroplets even if their spacing varied significantly. Being deposited together, the carrier fluid (typically a volatile perfluorinated oil) and the aqueous phase simply evaporated before the plate was loaded into the MALDI mass spectrometer. Evaporation of both phases is usually completed within less than 60 seconds or less than 5 seconds when a flow of nitrogen gas is used to accelerate the process. First tests of the spotting system and the control software were performed using a portable USB camera with high magnification for observation (see SI movie). Reliable spotting frequencies of up to 7 Hz were achieved.

For analysis by MALDI-MS, co-crystallization with a matrix is required to achieve efficient desorption and ionization of the analyte. One possibility is to inject the matrix solution into the aqueous stream prior to droplet creation. However, the addition of a highly acidic and saturated MALDI matrix solution at such an early stage can interfere with the assays performed inside the microdroplets. To circumvent this problem, the MALDI matrix can be deposited before or after

the deposition of the analyte. The matrix solution then re-dissolves the dried analytes (or vice versa) and leads to co- crystallization. Our system readily provides these possibilities as microdroplets containing the MALDI matrix solution can be created and deposited in the same way as the analyte solution.

Next, we proved the cross-contamination free spotting of droplets on the microarray. The hydrophobic coating of the plate prevented cross-contamination between adjacent hydrophilic spots and confined each droplet to a defined position within the array. To demonstrate this, droplets of an aqueous solution of a blue food dye (patent blue V) were spotted on the microarray in a vertical serpentine movement periodically omitting vertical lines to preserve empty spots (Figure S-2). Figure 2a shows a micrograph of a small section of the microarray after spotting the dye. No leakage of the aqueous phase into the adjacent empty hydrophilic spots nor spreading over the hydrophobic area could be observed by brightfield microscopy. After evaporating the oil phase and the analyte droplets, additional droplets of 2,5-dihydroxybenzoic acid (DHB) (MALDI matrix) were spotted in a second run across all spots in horizontal lines (Figure S-2) to test for unwanted transport of analyte between adjacent spots during matrix deposition. As the flow of perfluorinated oil between successive matrix droplets prevents any direct aqueous connection between successive spots, no carryover of dye could be observed. Furthermore, the absence of cross-contamination was also verified by MALDI-MS imaging (Figure 2b & d). Signals for patent blue V were obtained exclusively on the spots where droplets with the dye were deposited beforehand, while signals for DHB were obtained from every hydrophilic spot as expected. Since patent blue V can readily be ionized and detected even without applying MALDI matrix, the spreading of aqueous liquid over hydrophobic areas would have been visible in the MALDI-MS imaging experiment even in the absence of the matrix. For

this test of cross-contamination, the application of MALDI matrix was merely performed to detect any carryover effect of the analyte rather than to improve its ionization.

We demonstrate the potential of the system by monitoring the enzymatic conversion of angiotensin I (a decapeptide) to angiotensin II (an octapeptide) by angiotensin converting enzyme (ACE). This enzymatic conversion is an important step in the renin–angiotensin–aldosterone system (RAAS) that regulates blood pressure in humans. For the experiment, substrate (angiotensin I, 10 μg/mL) and enzyme (ACE, 5.35 units/mL) were mixed in an aqueous solution and directly loaded into a glass syringe. Using a flow rate of 0.25 μL/min for the enzymatic reaction mixture and 0.75 μL/min for the oil phase, droplet creation and spotting was started immediately upon mixing. Over a 3-hour period, 8,265 droplets of 3 nL containing the current reaction mixture were spotted at 0.7 Hz and immediately dried using a flow of dry nitrogen gas to quench the enzymatic reaction. Hence, each spot represents a single time point of the enzymatic conversion. The overall volume of spotted sample is only about 24 μL. Upon completion of spotting the enzymatic reaction, droplets of DHB were created and deposited on all spots.

After measuring each spot by MALDI-MS, the peak areas for angiotensin I and angiotensin II were extracted from each individual spectrum (see SI). The peak area ratio (angiotensin II/[angiotensin I + angiotensin II]) was then calculated for each spot to measure the progress of the reaction. Figure 3a shows a heat map of the peak area ratio for each spot in the microarray. Spots where no signal could be obtained are marked in black. The video analysis of the spotting of the enzymatic reaction and the subsequent spotting of the MALDI matrix revealed only occasional spotting errors. In total, a small fraction (under 1 % after both runs) of all spots did not yield a signal. Figure 3b shows the peak area ratio as a function of time and two exemplar

mass spectra taken from the start and the end of the monitoring process. The increase of the product is clearly visible and demonstrates that our interface facilitates droplet content detection, here additionally assessing the ratio of product to substrate in an enzymatic reaction over time.

The micro-array plate can be used as a sample archive on which multiple experiments can be stored and measured at a later time. Additionally, spotted droplets can be re-measured multiple times while switching the spectrometer between different acquisition methods (e.g. positive and negative ionization or between MS and MS/MS) (data not shown). This enables the analysis and identification of analytes even in complex samples.

In summary, we have developed an automated platform that enables fast deposition of individual microdroplets on a high-density array plate, which is directly compatible with standard MALDI mass spectrometers for label-free analysis of individual microdroplets. Although the spotting procedure, in principle, can be realized on non-patterned surfaces, the hydrophilic spots significantly increased the robustness and speed of the system, and allowed reproducible droplet spotting on the same position. Moreover, further downscaling and adaptation for the deposition of ultrasmall droplets down to picoliter volumes is possible.

Since the interface presented here uncouples the continuously operating microfluidic system from the mass spectrometer, droplets can be created and deposited at rates exceeding the processing speed of the spectrometer. Hence, our system also overcomes the problem associated with the hydrophobic carrier phase that typically compromises measurements with other common MS methods as it can be easily removed by evaporation.

We believe that the interface will widen the scope of microdroplet-based systems especially in the areas of combinatorial chemistry and drug discovery as well as other high-throughput screening applications such as single-cell proteomics or metabolomics.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

* Prof. P. S. Dittrich, Laboratory of Organic Chemistry, ETH Zürich Wolfgang-Pauli-Strasse 10, 8093 Zürich (Switzerland), E-mail: dittrich@org.chem.ethz.ch, Homepage: http://www.dittrich.ethz.ch.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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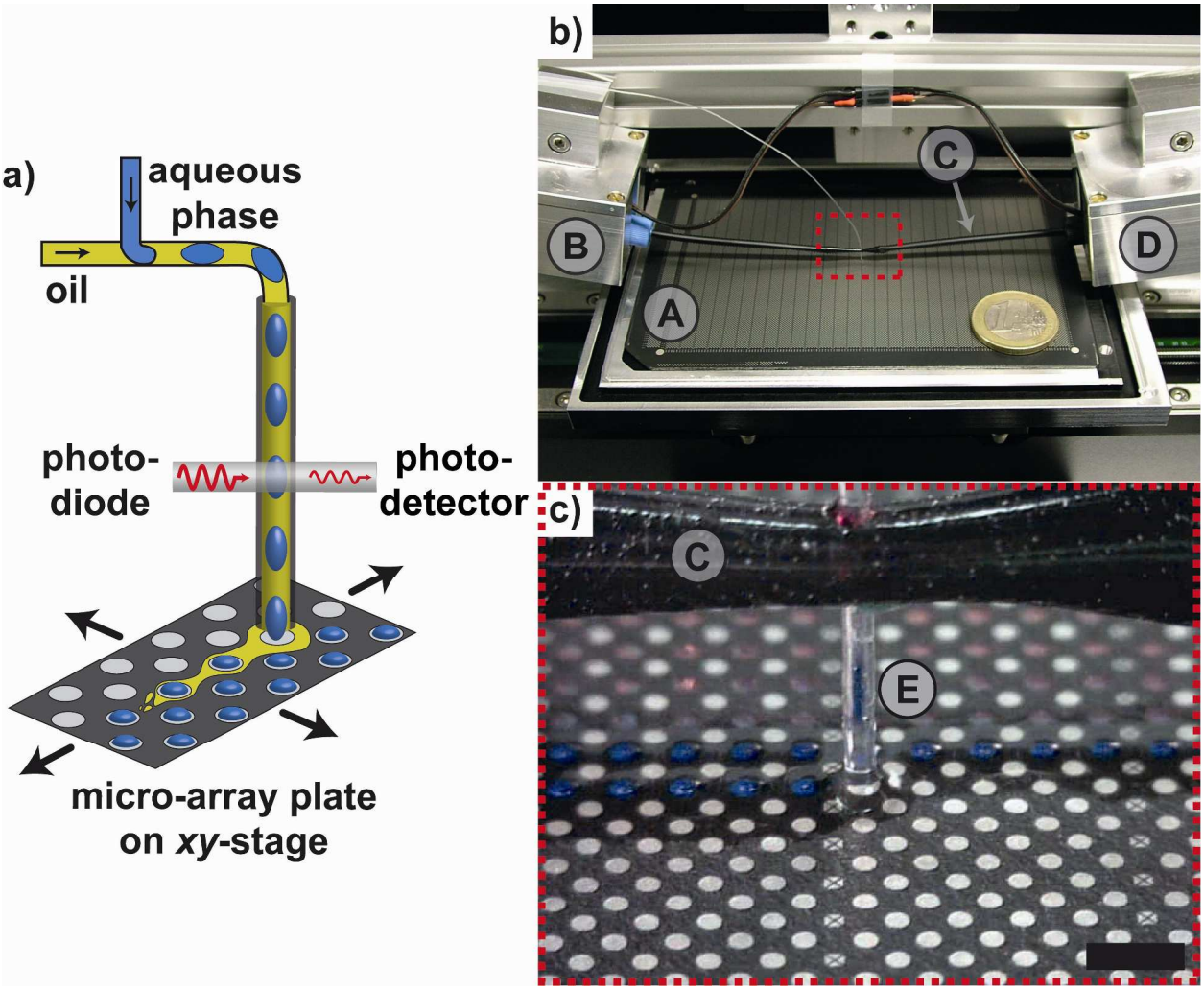

Figure 1. a) Schematic of droplet creation and spotting. The light areas on the plate indicate the hydrophilic spots on which the aqueous droplets are deposited. The hydrophobic carrier (oil) evaporates quickly upon deposition. b) Photograph of the interface showing the high-density array plate (here with a total of 26,444 hydrophilic spots of 300 µm diameter) mounted on an *xy*-stage (A) and the droplet detection setup consisting of a photodiode (B), an optical fiber (C) and a photodetector (D). c) Enlargement of b) showing the tip of the spotting capillary (E) and spotting of 3 nL droplets containing blue food dye on the hydrophilic spots. Scale bar: 1 mm.

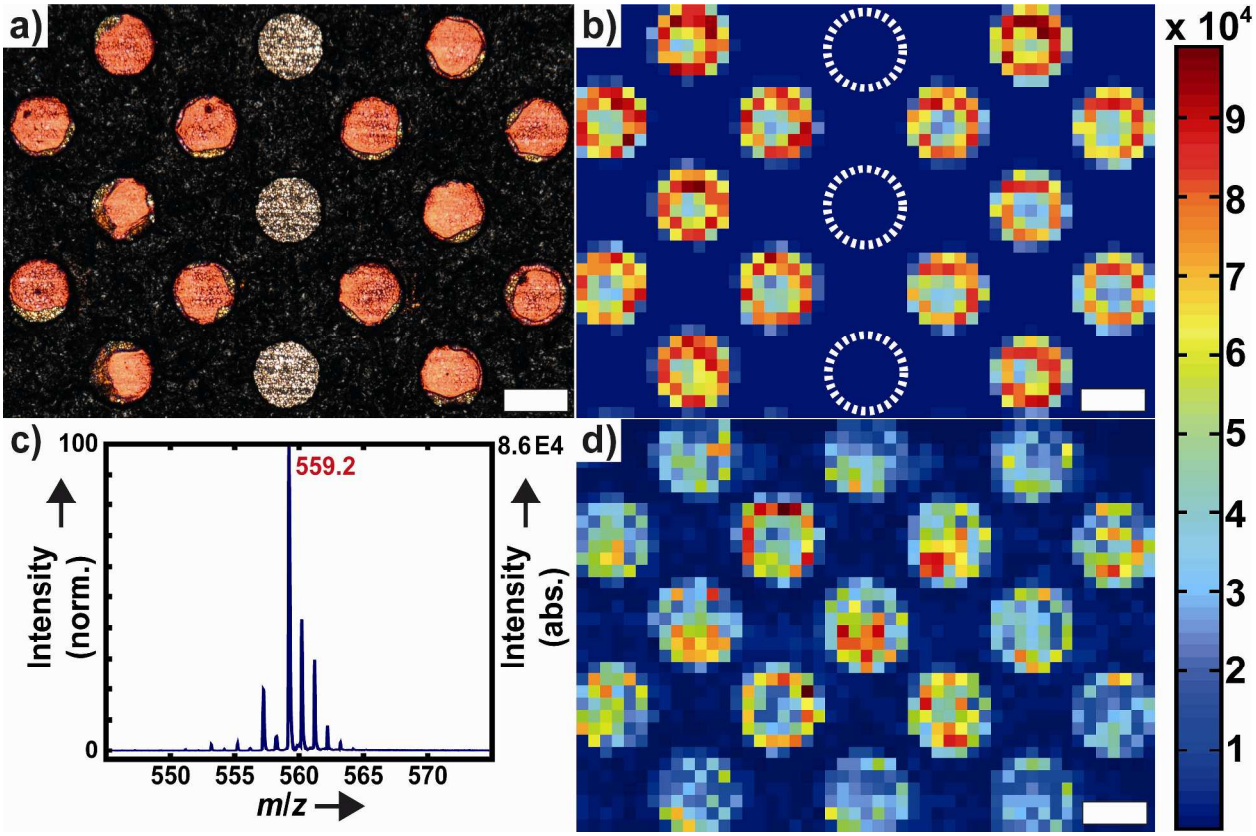

Figure 2. a) Micrograph showing a section of the microarray with food dye droplets (dried) deposited in vertical lines leaving one column empty. b) MALDI-MS imaging. After spotting DHB in horizontal lines, the microarray section showed in a) has been raster-scanned using 50 µm steps. The color indicates the intensity of the patent blue V peak at 559.2 *m*/*z* in the recorded MALDI-MS spectrum (c) of each point. d) The corresponding experiment for DHB. No cross-contamination of analyte could be observed. Scale bars: 300 µm.

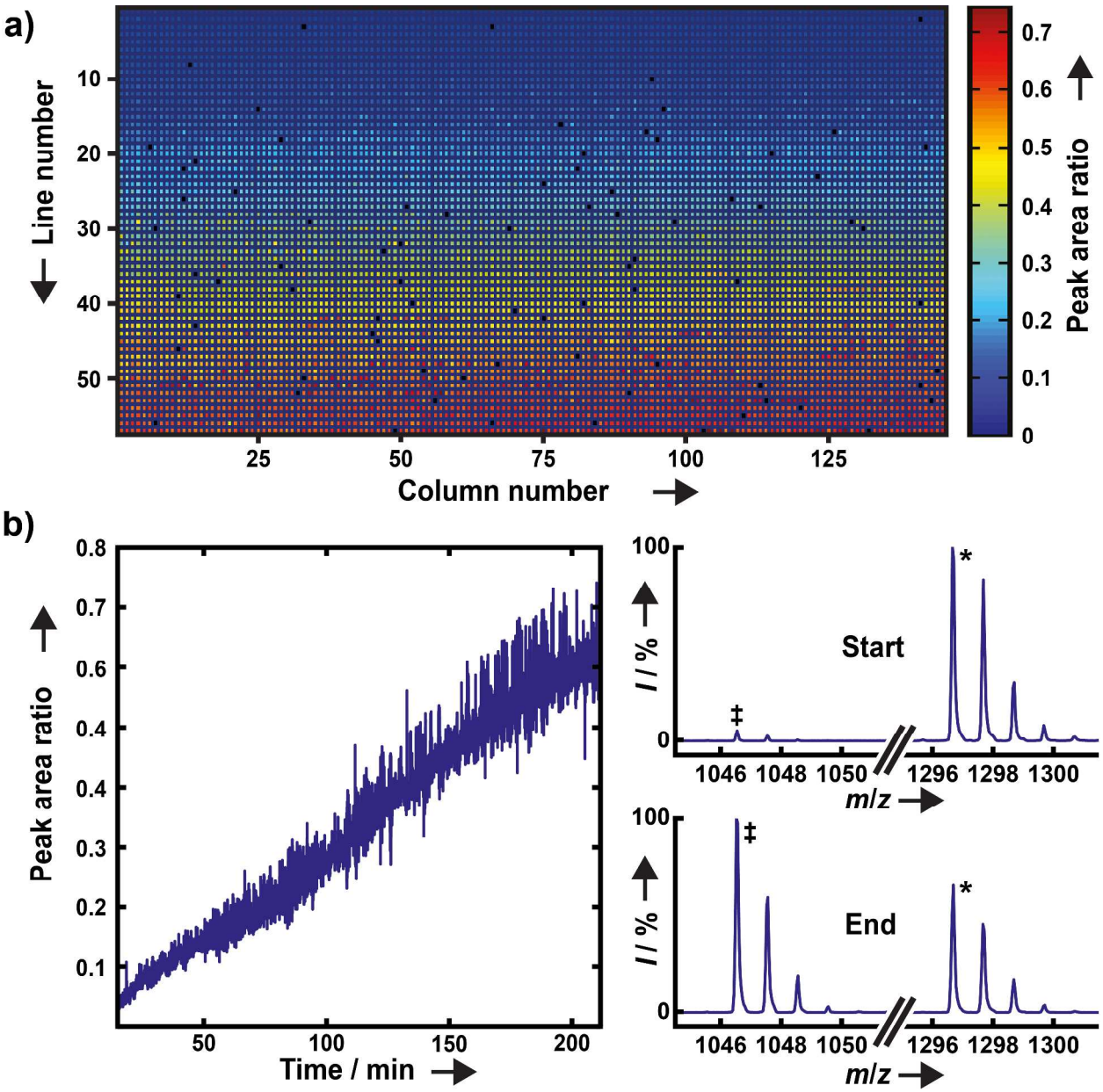

Figure 3. a) Heat map showing the peak area ratio of angiotensin I (substrate) and angiotensin II (product) (product/[substrate+product]) for the 8,265 droplets deposited on individual spots measured by MALDI-MS. b) Evolution of the peak area ratio over several hours and MALDI-MS spectra from the start and the end of the monitoring process (* angiotensin I, substrate; ‡ angiotensin II, product).