# Microfluidic carbon-blackened polydimethylsiloxane device with reduced ultra violet background fluorescence for simultaneous two-color ultra violet/visible-laser induced fluorescence detection in single cell analysis

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In single cell analysis (SCA), individual cell-specific properties and inhomogeneous cellular responses are being investigated that is not subjected to ensemble-averaging or heterogeneous cell population effects. For proteomic single cell analysis, ultra-sensitive and reproducible separation and detection techniques are essential. Microfluidic devices combined with UV laser induced fluorescence (UV-LIF) detection have been proposed to fulfill these requirements. Here, we report on a novel microfluidic chip fabrication procedure that combines straightforward production of polydimethylsiloxane (PDMS) chips with a reduced UV fluorescence background (83%-reduction) by using PDMS droplets with carbon black pigments (CBP) as additives. The CBP-droplet is placed at the point of detection, whereas the rest of the chip remains transparent, ensuring full optical control of the chip. We systematically studied the relation of the UV background fluorescence at CBP to PDMS ratios (varying from 1:10 to 1:1000) for different UV laser powers. Using a CBP/PDMS ratio of 1:20, detection of a 100 nM tryptophan solution (S/N = 3.5) was possible, providing a theoretical limit of detection of 86 nM (with S/N = 3). Via simultaneous two color UV/VIS-LIF detection, we were able to demonstrate the electrophoretic separation of an analyte mixture of 500 nM tryptophan (UV) and 5 nM fluorescein (VIS) within 30 s. As an application, two color LIF detection was also used for the electrophoretic separation of the protein content from a GFP-labeled single *Spodoptera frugiperda* (Sf9) insect cell. Thereby just one single peak could be measured in the visible spectral range that could be correlated with one single peak among others in the ultraviolet spectra. This indicates an identification of the labeled protein $\gamma$-PKC and envisions a further feasible identification of more than one single protein in the future. © *2012 American Institute of Physics*. [doi:10.1063/1.3675608]

## I. INTRODUCTION

The improvements over the past few years of microfluidic devices and lab-on-a-chip (LOC) systems demonstrate the beneficial integration of sensitive, reproducible and rapid separation, and detection techniques.[1–3] This potential was demonstrated by the transfer of numerous analyzing methods to the microfluidic format such as gel electrophoresis, isoelectric focusing, and corresponding detection techniques, e.g. electrochemical detection. Moreover, microfluidic systems allow investigation and analysis even of single cells (see, e.g., the work of Li *et al.*[4] and Huang *et al.*[5]) that cannot be achieved with traditional cell studies at the population level of $10^5$–$10^7$ cells.[6] The difficulties of such ensemble studies are averaging effects

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from cell-cycle-dependent states and inhomogeneous cellular responses on external stimuli.[6–8] Nevertheless, quantitative measurements of proteins are essential for understanding cellular behaviors and regulation mechanisms,[9] thus label-free detection techniques are desirable. In order to separate and analyze label-free biological compounds like proteins from single cells, microfluidics provides an effective and sensitive investigation tool in combination with UV laser induced fluorescence (UV-LIF) detection.[10,11] Promising alternative detection systems are integrated optical waveguides and electrochemical detectors for direct sensing in microchannels.[12] Therefore, Shadpour *et al.* reported a limit of detection (LOD) of 3.3 $\mu$M for proteins evaluated with the first multichannel microchip electrophoresis (MCE) device with integrated contact conductivity measurements.[13]

Because of the ambitious challenge to analyze a complex protein mixture in the minute volume of a single cell lysate,[14] to date mostly single or very few cellular components were investigated and analyzed. Exemplarily, Lapainis *et al.* demonstrated the detection of neurotransmitters,[15] or Wu *et al.* reported the electrophoretic separation of amino acids after cell lysis.[16] Furthermore, quantitative copy numbers of phycobiliproteins (as low as 400 molecules per cell), were obtained via single molecule fluorescence detection.[17] Nevertheless, proteins are difficult targets for single cell analysis (SCA) because (1) for proteins molecular amplification is actually not available and (2) identification of protein species is not trivial.[9] A coupling of microfluidic devices to mass spectrometry (MS) techniques is a promising approach to gain more information, and several MS based concepts have already demonstrated their potential.[18,19]

The necessary sensitivity range to detect low abundant proteins with copy numbers of $<10^5$ molecules per single cell[20] can be derived from considering a cell with a volume of 1 pl (diameter $\sim$10 $\mu$m) giving a limit of detection of $\sim$100 nM. In capillary electrophoresis LOD in the pM range for label-free LIF detection has been reported by Yeung and co-workers in 1992 using an $Ar^+$-laser ($\lambda_{ex} = 275$ nm).[21] With a metal vapor laser[22] or an excimer laser[23] detection limits in the nM range have also been published for analysis of the amino acid tryptophan. Besides tryptophan, the aromatic amino acids tyrosine and phenylalanine also exhibit intrinsic fluorescence, but for label-free UV-LIF detection, the fluorescence of native proteins is predominantly based on tryptophan.[24]

Concerning UV-LIF detection in microfluidic devices the choice of the chip material is very important and essential for the potential detection sensitivity. There is a need for UV-transparent materials that are not luminescent in the spectral region between 200 and 400 nm.[11] Ultrapure synthetic quartz glass (fused silica, FS) offers the most favorable characteristics. By using full body quartz glass chips, Greif *et al.* were able to reduce the theoretical LOD for tryptophan down to 2.5 nM.[10] However, due to its simple and low-cost processing for disposable devices,[11] polydimethylsiloxane (PDMS) as an alternative material for the fabrication of microfluidic devices yields a number of advantages. Unfortunately, conventional PDMS formulations often suffer from relatively high autofluorescence intensities around 360 nm, making useful and sensitive applications in UV-LIF detection a challenging task. In a recent technical note, UV light scattering in PDMS devices was reduced by the absorption of scattered UV light via shifting it to longer wavelength by loading the PDMS with a fluorescent dye.[25] This might be an interesting alternative to reduce the detectable UV fluorescence background in PDMS microchip applications.

In this paper, we focus on the improvement of PDMS microfluidic devices in order to decrease the autofluorescence background signal in the UV spectral range. Based on our previous studies of UV-LIF detection and with respect to our prior experimental enhancements by adjusting laser power, pinhole size, and separation buffer properties (especially concerning dynamic surface coatings),[10,26] we report here the application of a droplet of PDMS with carbon black pigments (CBP) as additive placed only at the point of detection keeping the rest of the chip transparent. Compared to previous attempts with fully carbon-blackened PDMS chips,[26] optical control in chip handling and therefore the possibility to conduct SCA can be maintained. In order to quantify the effect of the CBP-additive, the fluorescence background intensities were measured for different PDMS/CBP ratios. By simultaneously detecting

fluorescence signals in the UV and VIS spectral range, we demonstrate the electrophoretic separation of an analyte mixture of fluorescein and tryptophan. As an outlook and proof of concept, an UV/VIS single cell electropherogram of a lysate of a Sf9 insect cell is presented, where the detection of a distinct green fluorescent protein (GFP) labeled protein ($\gamma$-PKC) in the UV- and VIS-spectral channels is measured and discussed.

## II. EXPERIMENTAL

### A. Chemicals and reagents

PDMS (Sylgard 184 silicone elastomer and curing agent) was obtained from Dow Corning (Midland, USA). Fused silica (FS) cover slides (60 mm × 24 mm × 0.2 mm) were purchased from Hellma Optics (Germany). Pullulan, methylcellulose (MC), tryptophan, and fluorescein were obtained from Sigma-Aldrich (Germany). Tris(hydroxymethyl)aminoethane (Tris), dodecyl-beta-D-maltoside (DDM), (cyclohexylamino)ethanesulfonic acid (CHES), and phosphate-buffered saline (PBS) were purchased from Fluka (Germany). SU-8 (25) negative photoresist was obtained from MicroChem Corp. (USA) and mr-Dev 600 developer from Micro Resist Technology (Germany). CBP and tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorsilane (TTTS) were from ABCR (Germany). *Spodoptera frugiperda* (Sf9) insect cells and BacVector Insect Cell Medium were purchased from Novagen (Germany). Within these Sf9 cells, the protein $\gamma$-PKC (protein kinase C) was labeled with green fluorescent protein (GFP).[27,28] For all solutions deionized water from a Milli-Q biocel (Millipore, USA) was used.

### B. Chip fabrication

The PDMS microfluidic device is fabricated via soft photolithography[29] (see Fig. 1). First, a master wafer with the inverted relief of the microstructure made of SU-8 is produced.[30] To manufacture the blackened PDMS (bPDMS) droplets, the curing agent was initially well mixed with the CBP before the PDMS polymer (Sylgard 184) was added in a ratio of 1:10. This order is important for an easy and most efficient stirrings procedure. With a glass pipette several droplets of the uncured bPDMS were directly placed on the master wafer at the point of LIF detection, while the wafer was heated up to 100 °C on a heating plate. The diameter and the final height of the droplet are approximately 1 mm. Thereafter normal PDMS was poured over the wafer (Fig. 3, inset). After curing at 85 °C for 4.5 h, the PDMS layer was peeled of the wafer, and reservoir holes were punched for fluid access. The PDMS slabs were treated by oxygen plasma for 30 s in a home built glow discharge unit,[31] before assembly with the FS cover slide. To enlarge the volume of the reservoirs an additional 2 mm thick PDMS sheet with corresponding access holes was sealed on top of the completed chip.

A series of different ratios of CBP to PDMS was tested (from 1:10 to 1:1000; CBP to PDMS plus curing agent (w/w)) in the UV spectral range. For analyte separation and single cell experiments, a ratio of 1:20 was chosen. The chip design was adapted from Ref. 10 and consisted of a cross injector. In case of single cell experiments, the cross section area features rectangular obstacles (PDMS), acting as a mechanical cell trap (Fig. 1(f)).

Our PDMS data were referenced against data from a quartz glass microchip fabricated as described in Ref. 10.

### C. Fluorescence detection

Fluorescence detection of analytes and proteins after electrophoretic separation and CBP studies were performed on an inverted microscope (Axiovert 100, Zeiss, Germany) using two color UV/VIS-LIF detection (see Fig. 2). For excitation, a frequency quadrupled Nd:YAG laser (266 nm, 5 mW, Nanolase, France) and an argon-ion laser (488 nm, 5 mW, 161 C-010, Spectra Physics, Germany) are coupled via several mirrors into the rear port and the base port of the microscope, respectively. Both wavelengths were focused by an UV transparent objective (40×, Optics for Research, USA) into the microchannel superimposing at the same spot. Reduction of the UV laser power was realized by a gradient grey filter (LCVRQ-75 (0-3 nD), Laser

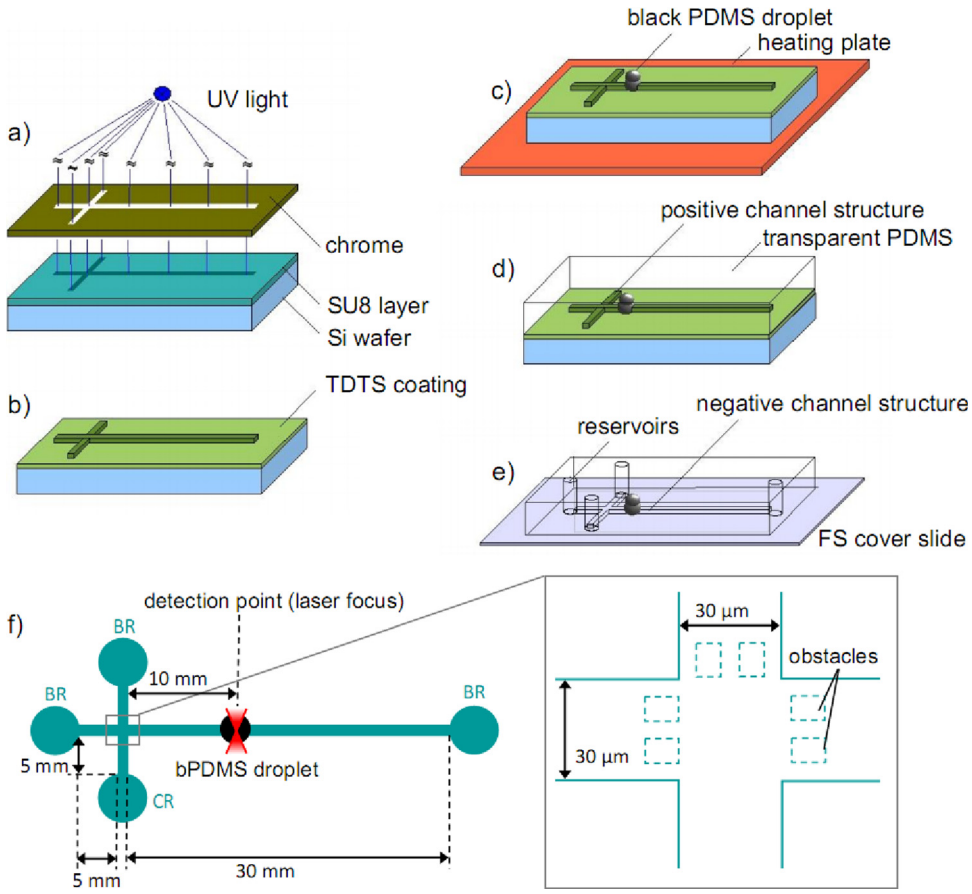


FIG. 1. Fabrication process of the bPDMS chips via soft photolithography. (a)/(b) Production of the master wafer. (c) Placing of blackened PDMS droplets directly on the heated master wafer at the desired point of LIF detection. (d) Molding of the microstructure via transparent PDMS. (e) Assembled microfluidic device with punched reservoirs on top of a FS cover slide. (f) Schematic layout of the cross injector with buffer reservoirs (BR) and the cell reservoir (CR). The diameter of the bPDMS droplet is approximately 1 mm and the diameter of the UV laser in the focus was calculated to be 3.4 $\mu$m. The inset shows the cross section area with the obstacles (size: 6 $\mu$m × 8 $\mu$m) for single cell trapping. The obstacles are made of PDMS and are fully spanning the height of the microchannels (30 $\mu$m).

Components, Deutschland) to 200 $\mu$W at the point of detection. For exact positioning of the excitation and detection window, a motorized x/y-stage (Märzhäuser, Germany) was used. Collection of the emitted fluorescence light was applied by the same objective. The optical path of the UV light consisted transmission through the first dichroic mirror (HR266 HT 300), reflection by a second dichroic mirror (475 DCX RUV, AHF Analysetechnik, Germany), spectral filtering by an interference filter (360/50, AHF, Germany) and focusing by a high transmission UV lens (G063342000, Linos, Germany) onto a commercial photomultiplier built-in module (H6240, Hamamatsu, Japan). The emitted fluorescence light in the visible spectral range passed through both dichroic mirrors mentioned before and a third one (BS 510, AHF, Germany), before it was spectrally filtered by an emission filter (LP 520, AHF, Germany) and focused by a lens (G063144000, Linos, Germany) onto a high sensitive avalanche photo diode (SPCM-AQRH, Perkin Elmer, USA). For maximal signals, both detectors can be positioned by x/y adjustable holders.

### D. Cell culture

The labeled Sf9 cells were grown to confluence of the first layer in BacVector Insect Cell Medium (Novagen, Germany) at 27 °C and 90% humidity. Prior to resuspending the cells in

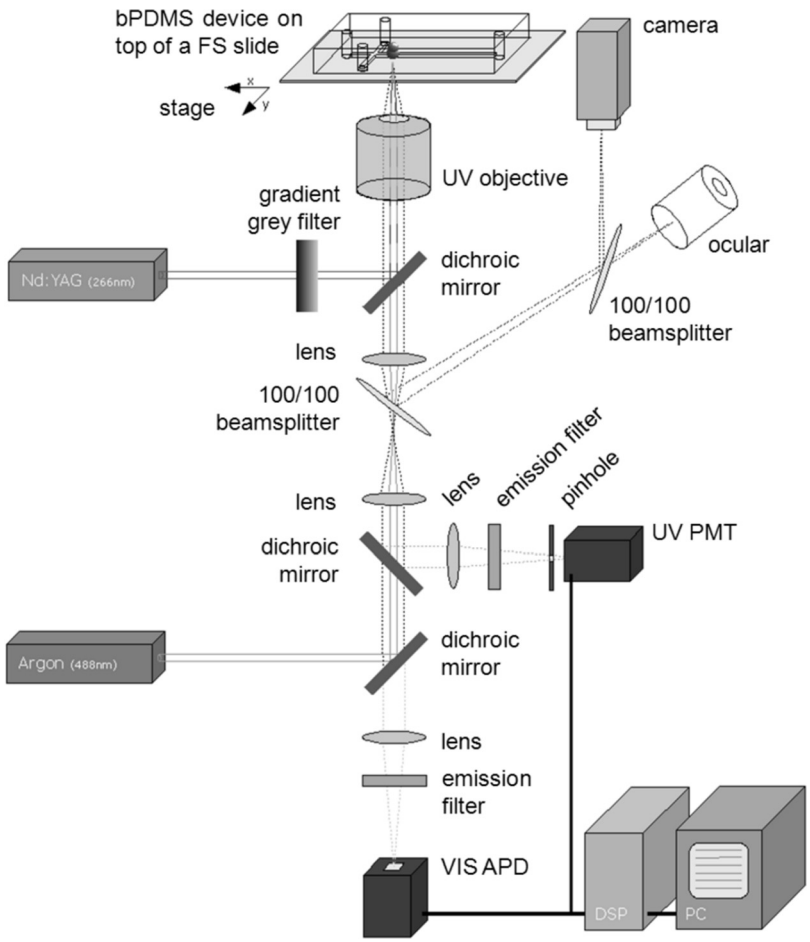


FIG. 2. Schematic diagram showing the basic components of the two color LIF detection setup. A frequency quadrupled Nd:YAG laser (266 nm) and an argon-ion laser (488 nm) were used for simultaneous excitation. Fluorescence detection was applied via a photomultiplier (UV) and an avalanche photo diode (VIS).

separation buffer and analysis on the chip, the cells were washed three times with PBS buffer to remove secreted proteins and residual impurities from the medium.

### E. Chip operations

Initial filling of the microchannels was done either by applying vacuum to an opposite reservoir or by capillary action. Previous to each experiment, the channels were washed with NaOH and MilliQ water. The systematic study with different CBP to PDMS ratio droplets, and the analyte injection and separation experiments were performed with Tris buffer (10 mM, *p*H 9.5). For single cell experiments, a separation buffer (100 mM Tris, 100 mM Ches, 8% Pullulan, *p*H 9.0) and 0.1% (w/w) DDM as dynamic coating were used. Voltage was applied via two or four platinum electrodes dipped into the reservoirs and connected to power suppliers from FUG (HCN 14-12500 and HCN 7 E-12500, Rosenheim, Germany). Data acquisition and instrumental control were executed by software programmed in LABVIEW (National Instruments, USA). For analyte injection and separation, the pinched injection method was used. Single cell experiments were performed as previously published[10,20] and can be briefly divided into four steps. After isolating and guiding a single cell of interest to the intersection position of the microdevice via hydraulic pressure (comparable to Ref. 5), the single cell was physically trapped by obstacles (Fig. 1(f)). Then, cell lysis was achieved by a short electrical high voltage pulse (2500 V/cm for 100 ms). Directly after lysis, the cell content was injected into the separation

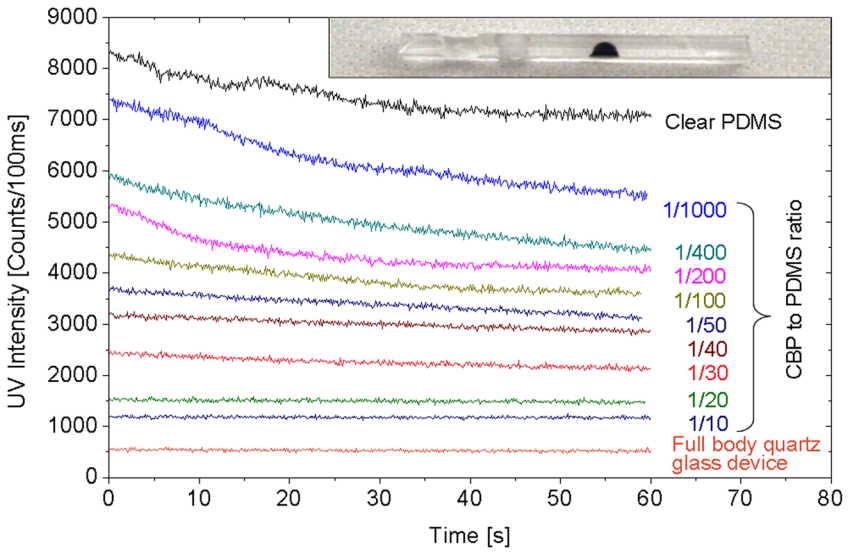


FIG. 3. Diagram showing the emission intensity ($\lambda_{em} = 335\,nm–385\,nm$) for different ratios of carbon black pigments detected below the bPDMS droplet in the microchannel for a time period of 60 s. The background fluorescence of clear PDMS and of a full body quartz glass device is also plotted for comparison. Excitation was performed at 200 $\mu$W laser power ($\lambda_{ex} = 266\,nm$). The microfluidic channels were filled with Tris buffer with *p*H 9.5. Inset: photograph of a cross section of a bPDMS droplet within a microfluidic chip for UV-LIF detection. The height of the blackened PDMS droplet is round about 1 mm and the radius 0.5 mm.

channel, followed by the separation of the different cell compounds with electric field strength of 225 V/cm. Simultaneously, UV and VIS LIF detection were performed at the detection point at a distance of 10 mm from the cross section.

## III. RESULTS AND DISCUSSION

### A. Background fluorescence of different PDMS to CBP ratios

Compared to earlier attempts with full carbon-blackened PDMS microfluidic devices on fused silica cover slides,[26] we improved the fabrication procedure in order to maintain optical transparency for chip handling in combination with strong reduction of the UV background fluorescence ($\lambda_{em} = 335\,nm–385\,nm$). We investigated the background signals of a microfluidic channel filled with Tris buffer at *p*H 9.5 over a time period of 60 s. Except for reference measurements of clear PDMS, the focus is in the center of the bPDMS droplet and in the middle of the microchannel (see Fig. 3, inset). Since the CBP are responsible for UV-radiation absorption, the level of UV-background fluorescence reduction strongly depends on the used ratio of PDMS to CBP (see Fig. 4). In contrast to the initial high fluorescence background intensity of clear PDMS (7094.5 counts/100 ms), a 83%-reduction in background signal could be observed for measuring under a bPDMS droplet (1174.4 counts/100 ms) with a CBP to PDMS ratio of 1:10, at 200 $\mu$W laser power (excitation), considerably approaching the normative UV background signal of a reference full body quartz glass chip (519.8 counts/100 ms). The corresponding noise of the background fluorescence intensity could be reduced by 71% (from $\pm$68.6 counts/100 ms to $\pm$20.1 counts/100 ms), reaching the favorable noise level observed from a full body quartz glass chip ($\pm$20.4 counts/100 ms) for the measurement as shown in Fig. 3. The respective average fluorescence background intensity was measured and integrated over a time period of the last 10 s where the standard deviation of the signal was taken as indicator for the corresponding noise. With these fluorescence background characteristics, the bPDMS microchips are — among other approaches such as previously published PDMS quartz window (PQW) chips[32] — a valuable alternative to full body quartz glass devices.

The reduction of the background fluorescence by the application of a blackened PDMS droplet is mainly caused by both the absorption of the UV laser, leading to low PDMS autofluorescence. A further minor contribution is due to a decreased autofluorescent PDMS volume.

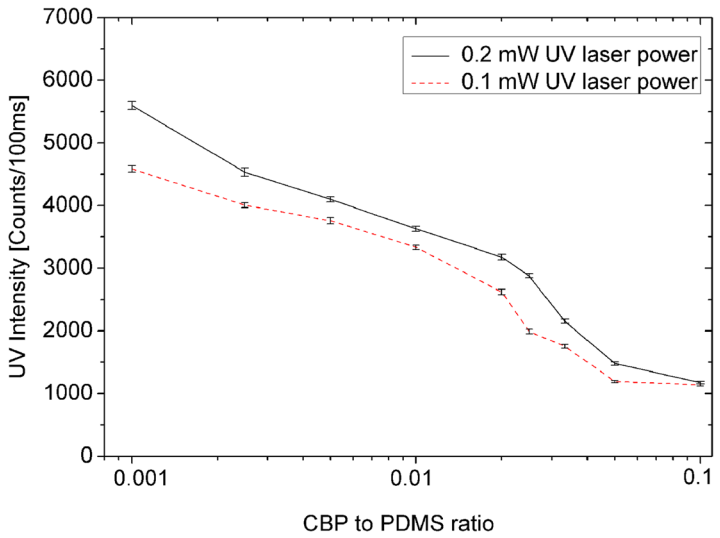


FIG. 4. Average emission intensity ($\lambda_{em} = 335$ nm–385 nm) over the time period of the last 10 s for different CBP ratios. The solid line represents 200 $\mu$W laser power and the dashed line 100 $\mu$W laser power for excitation ($\lambda_{ex} = 266$ nm). Error bars obtained from standard deviation.

In addition, the significant decrease of the fluorescence background bleaching to a negligible level, completely vanishing above a ratio of 1:20 (Fig. 3), is proved to be beneficial. This guarantees straightforward chip operation under optimized conditions without waiting time to reach a stable baseline.

Interestingly, laser power reduction from 200 $\mu$W to 100 $\mu$W does not lead to a significant decrease of the background signals (see Fig. 4). Thereby the intensity for the CBP/PDMS-ratios of 1:10 and 1:20 with 1138.4 counts/100 ms and accordingly 1191.6 counts/100 ms are at the same low level. In the following, a CBP to PDMS ratio of 1:20 is used for all following injections as well as for the single cell experiments.

### B. Analyte injection and separation

In order to determine the LOD in the ultraviolet spectral range for the novel bPDMS chips, Fig. 5 shows exemplarily an electropherogram of a 100 nM tryptophan injection. The analyte was pinch injected followed by native UV-LIF detection. The corresponding S/N (peak height/standard deviation) ratio is 3.5, which gives a theoretical LOD of 86 nM (S/N = 3). For the injection of a 1 nM fluorescein solution measured with VIS-LIF detection (data not shown), we obtain an S/N ratio of 4.7 with the associated theoretical LOD of 639 pM. Conclusively and in consideration of the envisioned single cell experiments with bPDMS chips, these detection sensitivities fulfill the requirements to detect low abundant proteins.[33]

According to the individual analyte injection and detection, we separated an analyte mixture of tryptophan and fluorescein followed by simultaneous two color UV/VIS-LIF detection. Fig. 6 illustrates an electrophoretic separation of an analyte mixture containing 500 nM tryptophan and 5 nM fluorescein. Though the two peaks originate from the same separation but different detection wavelengths, it is clearly visible that both analytes were separated with "baseline resolution" within 30 s.

### C. Single cell experiments

Consequently improving our work with label-free protein fingerprinting of single cells in microfluidic devices already published,[10,26,32] we aspire for an extension of our approach of label-free UV-LIF detection.

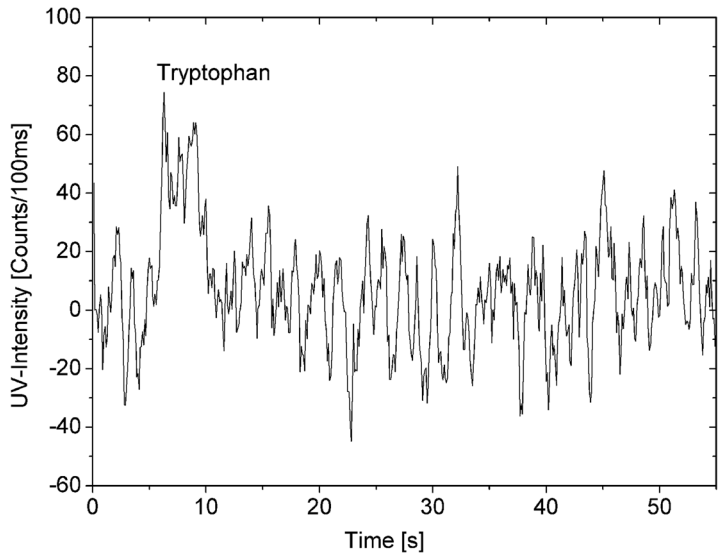


FIG. 5. Native UV-LIF detection ($\lambda_{ex} = 266$ nm) of a 100 nM tryptophan solution injected with an electric field strength of 242 V/cm (pinched injection; separation distance 10 mm; Tris buffer at *p*H 9.5). UV-LIF was performed at 200 $\mu$W laser power. Original data are baseline corrected and smoothed with a four point binning.

As an example, Sf9-insect cells distinctively expressing a single GFP-labeled protein were used ($\gamma$-PKC fused with GFP). A single cell electropherogram of a Sf9 cell obtained in a bPDMS microchip by two color UV/VIS-LIF detection is illustrated in Fig. 7. Dynamic coating of the microchannel walls with 0.1% (w/w) DDM was used for the reduction of electroosmotic flow (EOF) and diminished unspecific protein adsorption,[34] whereas pullulan acts as sieving matrix within the separation buffer. Performed SCA includes four steps: cell trapping, electrical induced cell lysis, electrophoretic separation of cellular protein content followed by LIF detection. In Fig. 7, one can clearly identify a distinct peak in the UV-spectrum which correlates in the time domain with the most prominent single peak in the visible spectral channel. This reproducible result is indicative of a perfect peak matching referring to the GFP-labeled fusion protein that can be detected in the UV- as well as in the VIS-channel. Interestingly, about

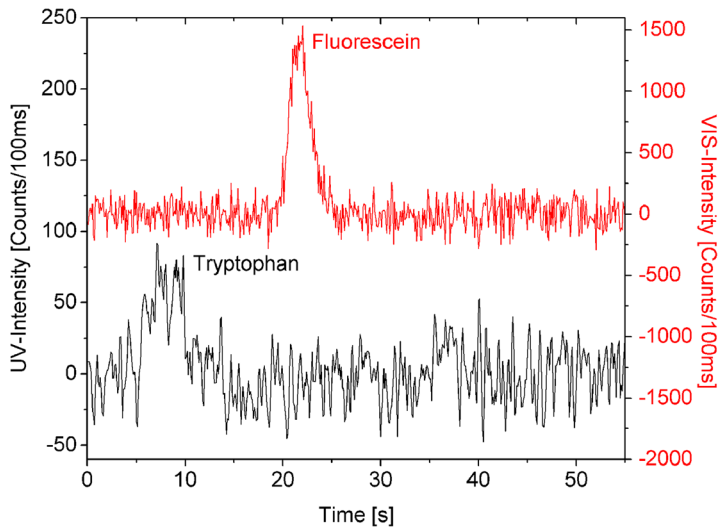


FIG. 6. Electrophoretic separation and two color LIF detection (UV: $\lambda_{ex} = 266$ nm, VIS: $\lambda_{ex} = 488$ nm) of a solution containing 500 nM tryptophan and 5 nM fluorescein. Injection parameters are the same as for the tryptophan injection in Fig. 5. Original data are baseline corrected and in case for the tryptophan electropherogram smoothed with a two point binning.

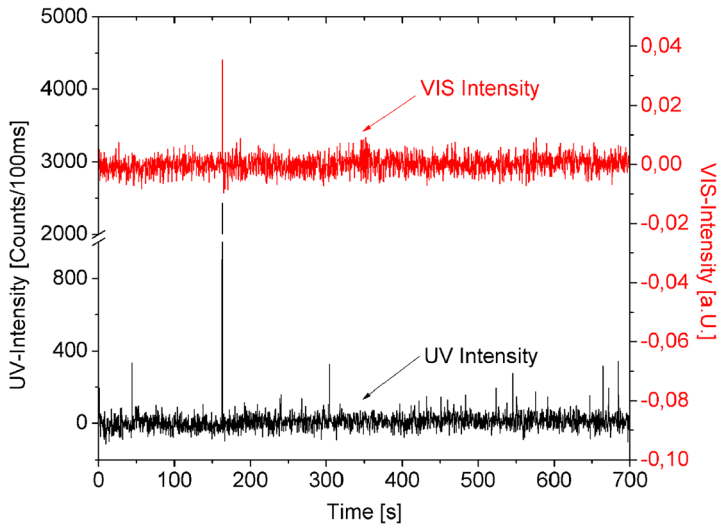


FIG. 7. Baseline corrected single cell electropherogram from a Sf9 cell expressing a GFP fusion protein with simultaneous two color UV/VIS-LIF detection (UV: $\lambda_{ex} = 266$ nm, VIS: $\lambda_{ex} = 488$ nm). The microchannels of the bPDMS-chips are coated dynamically with 0.1% DDM (w/w) at *p*H 9.0 of the separation buffer. After cell lysis (pulse: 2500 V/cm for 100 ms), a separation voltage of 225 V/cm was applied (detector at 10 mm). Exact chip layout and chip operations are specified in Sec. II.

10 other and smaller peaks can be observed in the UV channel, reflecting other (and unknown) protein species that can be detected by their autofluorescence properties but are invisible in the VIS-channel.

## IV. CONCLUSION

By the integration of a small droplet of carbon-black pigments into a transparent PDMS microfluidic device at the point of detection, it was possible to demonstrate an 83%-reduction of the UV fluorescence background signal, compared to the fluorescence signals of clear PDMS. Compared to standard (and expensive) quartz full-body chips, the measured fluorescence background signal of a disposable carbon-blackened PDMS microdevice that can be mass produced compares favorably where its respective signal-to-noise level already reaches the normative level of the fused silica system (61%-noise reduction compared to transparent PDMS). By the application of these bPDMS microchips detection of a 100 nM tryptophan solution was possible, giving a theoretical LOD of 86 nM. Integration of this experimental microdevice into a two-color LIF detection setup allows the simultaneous detection of fluorescence signals in the UV- as well as VIS-spectral range, as shown by a separation of an analyte mixture of 500 nM tryptophan and 5 nM fluorescein. Finally, we showed that the identification of the GFP fusion protein $\gamma$-PKC at the position of a UV peak by detection of the corresponding VIS peak within a single cell UV/VIS electropherogram was possible. In the future, detection of several different markers for different proteins or detecting distinct protein species of industrial relevance in single cells, e.g., for the production of therapeutic proteins by cell cultures,[35] are planned.

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