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## In-line whole blood fractionation for Raman analysis of blood plasma

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Blood plasma evaluation has high significance in clinical diagnostics. Current schemes involve the preparation of blood plasma by centrifugation of whole blood followed by electrochemical or spectroscopic analysis. However, centrifugation is often too time-consuming for application in clinical emergency and point-of-care settings. We propose to combine microfluidic, instantaneous plasma fractionation with localized spectroscopic methods for in-line analysis. As an example, we present confocal Raman spectroscopy in fractionated plasma domains at two different Raman excitation wavelengths. Resonance Raman spectroscopy with laser excitation at 408 nm allows the specific detection of free hemoglobin in blood plasma at concentrations above 22 mg dl<sup>-1</sup> (level of detection). Consequently, we are able to accurately resolve the range of clinical relevance regarding hemolysis. At near-infrared excitation (785 nm) we furthermore demonstrate the acquisition of characteristic Raman spectra of fractionated blood plasma in the microfluidic setting. These spectra can serve as starting point for a multi-parameter regression analysis to quantify a set of blood plasma parameters from a single Raman spectrum. The combined microfluidics and Raman spectroscopy method is non-destructive and has a whole blood consumption of less than 100 μl per hour. It thus allows for continuous in-line blood plasma monitoring.

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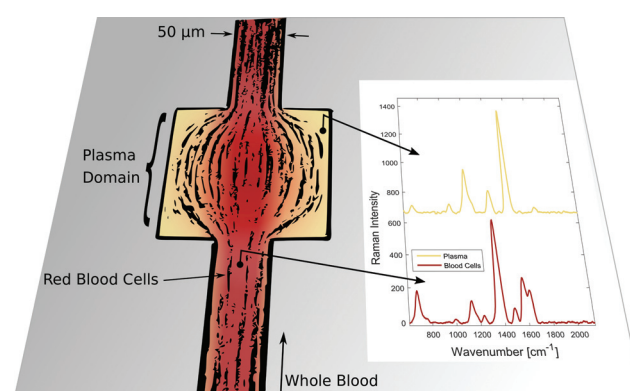
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## 1 Introduction

Exact and reliable blood assay is key in clinical diagnostics. Every day millions of blood samples are drawn from patients around the globe to provide physicians with crucial information on the patients' health status. Point-of-care blood testing, in particular in emergency settings, requires particularly fast sample throughput and analysis, on a timescale of less than one minute. In this study we demonstrate an in-line blood plasma analysis, where we employ optical sensing inside a locally expanded plasma domain in microfluidic whole blood flow. With this method, time-consuming centrifugation of whole blood is circumvented. Furthermore, continuous blood sample consumption can be kept below 100 μl per hour due to small microfluidic channel dimensions, thereby enabling continuous plasma monitoring or analysis in a prick test. Fig. 1 illustrates the non-permanent, yet instantaneous cell-free plasma domain creation alongside the boundaries of the microfluidic channel expansion. Confocal laser illumination on streaming red blood cells and on fractionated plasma results in distinct Raman spectra.

Blood flow in micro-capillaries and microchannels has been the subject of research for many years as it provides

information on *in vivo* blood flow in microcirculation.<sup>1,2</sup> Whole blood can be considered as a suspension of erythrocytes in a Newtonian fluid,<sup>3</sup> namely blood plasma. Due to the velocity gradient across a shear flow in a microchannel and the consequent hydrodynamical lift, deformable cells tend to migrate away from the channel boundaries towards the middle of the channel resulting in cell-free plasma layers adjacent to



**Fig. 1** Instantaneous blood plasma fractionation appears adjacent to the channel walls of a geometric expansion in microfluidic whole blood flow. Local Raman spectroscopy on plasma domains (orange) is different compared to domains of streaming whole blood (red) where Raman scattering originates from cellular hemoglobin.<sup>4</sup> The Raman excitation wavelength is 408 nm.

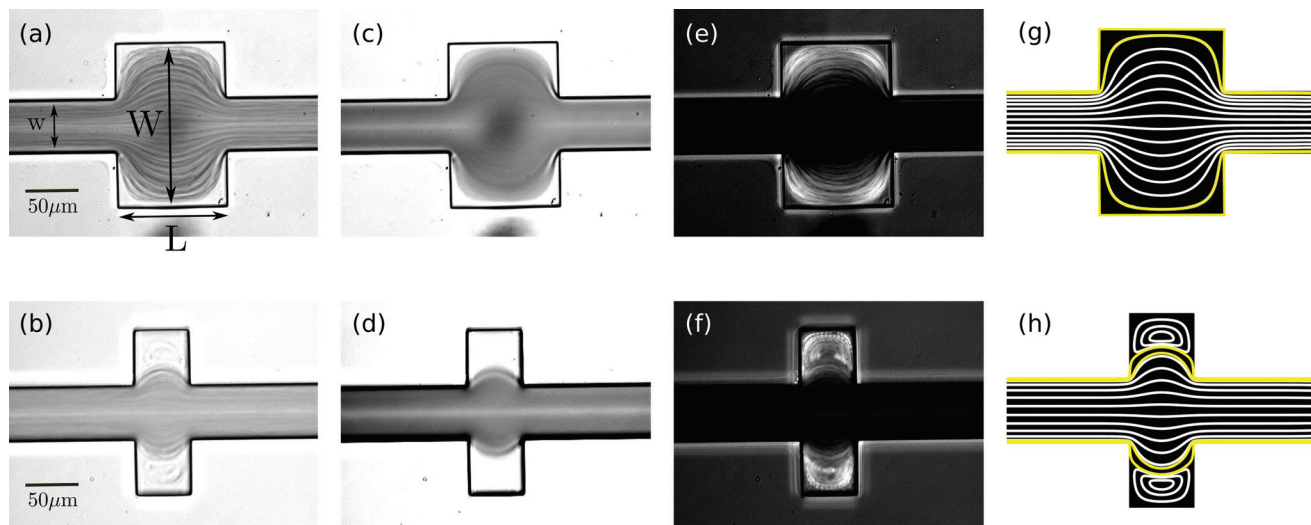
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**Fig. 3** Experimental microfluidic whole blood stream in 40  $\mu\text{m}$  deep PDMS channels and localized cell-free domain creation for two different expansion geometries. From left to right, respectively: 10 ms snapshot (a–b), time average of blood flow time series (c–d), standard deviation of blood flow time series (e–f) and creeping flow streamline simulation (g–h). The yellow streamlines indicate the extent of the plasma domain that is fed by the 1.5  $\mu\text{m}$  wide plasma layer of the inlet whole blood flow.

incompressible Newtonian fluid having identical properties to the plasma. The Reynolds number of our system is low ( $\text{Re} \approx 0.1$ ) so that inertial terms can be neglected and the 2D Stokes equation in the shallow channel approximation<sup>36</sup>

$$\mu \nabla^2 \mathbf{u} - \nabla p - \frac{12\mu}{h^2} \mathbf{u} = 0, \quad (1)$$

$$\nabla \cdot \mathbf{u} = 0 \quad (2)$$

applies as the governing equation in steady-state creeping flow simulations. Here  $\mathbf{u}$  is the in-plane velocity field,  $\mu$  is the dynamic viscosity of the liquid,  $\nabla p$  is the pressure gradient and  $h$  is the channel depth.

### 3 Results and discussion

#### Microfluidic instantaneous plasma domain creation

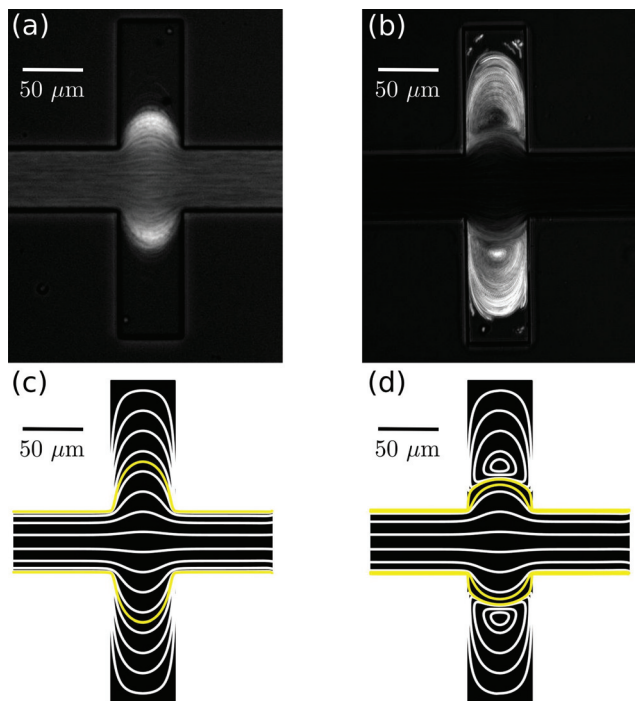
In a straight microfluidic channel, the plasma layer in the streaming blood is about 1.5  $\mu\text{m}$  wide.<sup>1,3</sup> This is too small for confocal probing under moderate numerical apertures. Therefore we aim to locally expand this cell-free layer into extended plasma domains of at least 10  $\mu\text{m}$  in diameter which can be achieved by introducing a local expansion in the otherwise  $w = 50 \mu\text{m}$  wide microfluidic channel. Fig. 3 illustrates that the patterns of blood flow and the extent of fractionated plasma domains in the expansion are determined by its geometry. We modify the length  $L$  of the expansion while expansion ratio  $W/w = 3$  and channel height  $h = 40 \mu\text{m}$  are kept constant. For  $L = 2w$  streamlines are continuous and sufficiently expanded plasma domains reach the corners of the expansion. In the case of  $L = w$  (lower row in Fig. 3), eddies occur – as analytically described by Moffat<sup>37</sup> – which implies that the incoming

plasma layer is not largely expanded in the corners of the expansion geometry. Nevertheless, the plasma layer feeds the eddy by diffusion across their common boundary. But due to the small width of the plasma layer inside the expansion, some cells enter the eddy and circulate. This effect becomes most obvious in standard deviation images of the blood flow time series.

Confocal laser illumination in the region of a microfluidic eddy is critical. Although this region contains blood plasma, some cells circulate and can get trapped and destroyed by the focused laser. Therefore we favor continuous streamlines throughout the entire channel expansion for in-line Raman measurements. In order to avoid the presence of circulating eddies, one can reduce the channel height  $h$ . Fig. 4 demonstrates both experimentally and numerically that a reduction of the channel height from  $h = 40 \mu\text{m}$  to  $h = 20 \mu\text{m}$  makes the circulating eddies disappear in case of an in-plane geometry with  $W/w = 5$ ,  $L = w$  and  $w = 50 \mu\text{m}$ . The cell-free blood plasma domains at the reduced channel height in Fig. 4(a) have a large extent of about 50  $\mu\text{m}$  by 50  $\mu\text{m}$  enabling exclusive plasma domain optical sensing. On the other hand, the reduction of the channel height implies that the optical interaction length for Raman scattering is reduced. However, the channel height  $h = 20 \mu\text{m}$  is well suited for Raman experiments with a 60 $\times$  air objective because the depth of the field is of the same order as the height of the microfluidic channel. The only drawback of too large plasma domains is the strongly reduced plasma velocity in the outer regions of the expansion compared to the center of the channel so that sample exchange here cannot be considered instantaneous.

The size of the cell-depleted domains is nearly constant at all underpressures we have applied in the range of 1–100 mbar. Only at low flow velocities at pressures below



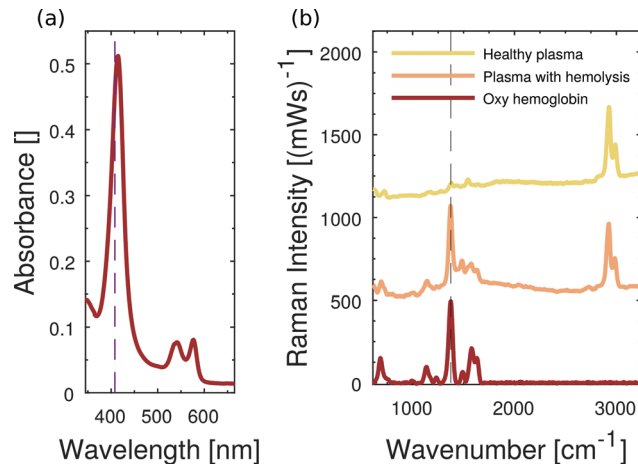


**Fig. 4** Different flow patterns for the same in-plane channel geometry, but different channel depths of  $h = 20 \mu\text{m}$  (a, c) and  $h = 40 \mu\text{m}$  (b, d). Panels (a–b) show the standard deviation of microfluidic experiments whereas panels (c–d) illustrate the streamlines of corresponding creeping flow simulations.

10 mbar, we observe a tendency towards smaller-sized blood plasma domains. If no underpressure is applied to the outlet of the microchannel, blood stands still and the geometric channel expansion is entirely filled with red blood cells, including the corners. At non-zero flow velocities, cell-free domains appear which is due to the stable hydrodynamical effect. Apart from fluctuations at the boundary, the outline of cell-free domains is constant with respect to time. The averaged images of two seconds long time series in Fig. 3 demonstrate the temporal stability of flow patterns. In fact, it is possible to maintain the same flow pattern for several hours in our setup.

### Raman hemoglobin evidence in bovine plasma

By choosing the Raman excitation wavelength of 408 nm, the incident light field is in resonance with part of a continuum of electronic transitions arising from the central and photoactive part of the hemoglobin molecule. The entire continuum is called the Soret band and becomes most apparent at about 420 nm in the hemoglobin absorption spectrum as shown for oxygenated hemoglobin in Fig. 5(a). The porphyrin structure of the heme group is highly Raman active, in particular, if both the resonant laser excitation and the Raman emission are located within its Soret band.<sup>24</sup> We see strong Raman scattering by oxygenated hemoglobin, in particular at  $1375 \text{ cm}^{-1}$  which corresponds to an emission wavelength of about 432 nm considering 408 nm Raman excitation. The reference Raman spectrum of oxygenated hemoglobin is shown at the



**Fig. 5** Resonance Raman spectroscopy of hemoglobin in liquids. (a) Absorption spectrum of oxyhemoglobin, the resonant laser excitation at 408 nm is spectrally located inside the Soret band. (b) Raman spectra of plasma without (yellow) and plasma with (orange) oxyhemoglobin in microfluidic channels. The most dominant Raman spectroscopic evidence of hemoglobin appears at  $1375 \text{ cm}^{-1}$ . The distinctive PDMS signature is spectrally located at about  $2900 \text{ cm}^{-1}$ . Spectra are vertically offset for clarity.

bottom of Fig. 5(b). In the following part, we focus on the Raman signature of oxygenated hemoglobin in liquid plasma solutions.

The microfluidic channel is filled either with healthy bovine plasma or with bovine plasma that carries dissolved oxygenated hemoglobin. The acquired Raman spectra of both pure plasma and hemoglobin-enriched plasma are plotted in Fig. 5(b). Beside characteristic Raman scattering, we observe a continuous fluorescent background from bovine blood plasma proteins. In the case of added hemoglobin, however, the characteristic Raman signature of oxygenated hemoglobin appears and superimposes the fluorescent emission. The resonance Raman scattering from hemoglobin is so strong that it is not hidden in the fluorescent background. The double-shouldered Raman band at about  $2900 \text{ cm}^{-1}$  originates from Raman scattering by C–H bonds of the microfluidic chip material PDMS.

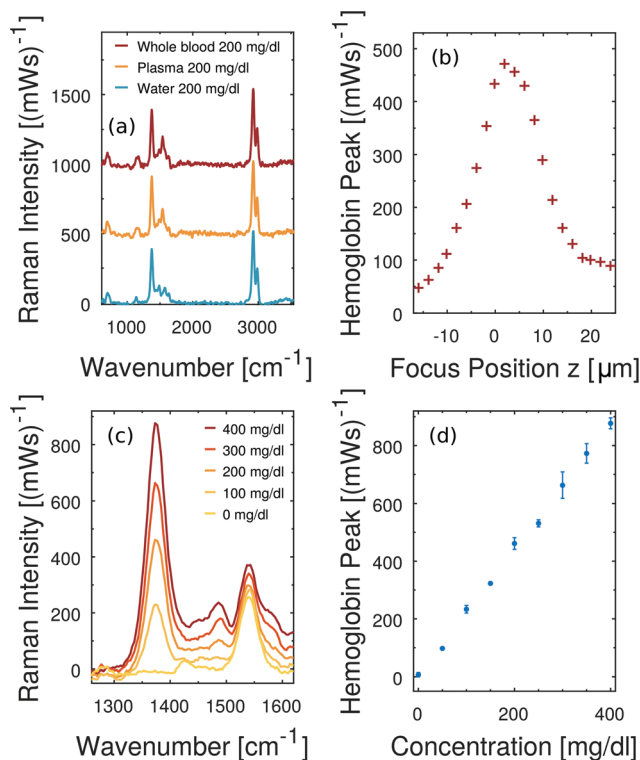
### Quantitative Raman analysis of free hemoglobin in instantaneously created blood plasma

Firstly, we demonstrate that the microfluidic in-line plasma separation locally creates blood plasma as demanded. Here we prepare both a centrifuged blood plasma sample with  $200 \text{ mg dl}^{-1}$  dissolved free hemoglobin and a whole blood sample with  $110 \text{ mg dl}^{-1}$  dissolved free hemoglobin. Considering the hematocrit of that particular whole blood being 45%, the plasma phase of this second sample has an effective free hemoglobin concentration of  $200 \text{ mg dl}^{-1}$  as well. The Raman excitation is directed into the dynamically created cell-free plasma domain of the whole blood sample in the microfluidic flow cell, as illustrated in Fig. 2(a). The same optical alignment is used, when the conventionally centrifuged plasma sample

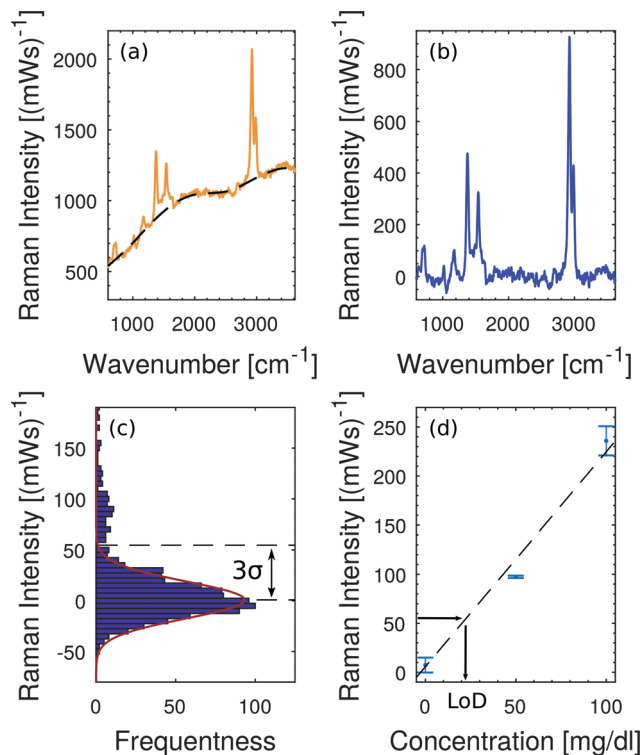


flows through the microchannel. In Fig. 6(a) we compare the baseline corrected<sup>38</sup> Raman spectra of these two samples and observe a perfect agreement. Furthermore, the equivalent concentration of free hemoglobin dissolved in water results in the same hemoglobin Raman peak intensity at  $1375\text{ cm}^{-1}$ . Minor differences between the latter and the plasma spectra – in particular in the spectral region at about  $1530\text{ cm}^{-1}$  – are due to the presence of beta-carotene in blood plasma samples.<sup>14,29</sup>

High consistency in hemoglobin Raman signal intensity enables quantitative free hemoglobin sensing. However, we note that reproducible scattering experiments are sensitive to the optical alignment. In particular, the small depth of field of our optical collection system and small channel depth imply that minor changes regarding the in-focus position  $z$  have a measurable effect on the detected Raman scattering. Fig. 6(b) illustrates the peak intensity of the smoothed hemoglobin signal at  $1375\text{ cm}^{-1}$  as a function of the focus position  $z$ . The channel is  $20\text{ }\mu\text{m}$  deep. The collected Raman intensity is not constant across this range demonstrating how important a stable position of the microfluidic channel with respect to the



**Fig. 6** Free hemoglobin measurements in fractionated whole blood flow. (a) Baseline corrected Raman spectra of fractionated whole blood, blood plasma and water, all carrying dissolved free hemoglobin through the microchannel. The mentioned concentrations refer to free hemoglobin in the plasma/aqueous phase. (b) The hemoglobin Raman peak intensity is sensitive to the alignment of the optical system. (c) Averaged baseline-corrected Raman spectra at different hemoglobin concentrations. The characteristic hemoglobin peak increases monotonically with increasing free hemoglobin content. (d) Raman peak intensity scales linearly in the hemolysis range of clinical relevance. In all Raman measurements we apply  $0.8\text{ mW}$  laser power at the sample and 3 seconds integration time.



**Fig. 7** Baseline fitting and level of detection (LoD). (a) Raw spectrum at  $200\text{ mg dl}^{-1}$  hemoglobin in plasma and the corresponding baseline fit<sup>38</sup> which accounts for the fluorescent background. (b) Baseline-corrected Raman spectrum. (c) Noise distribution close to the baseline fit. The LoD is defined as  $3\sigma$  of this noise distribution. (d) The concentration calibration from Fig. 6(d) translates  $3\sigma = 52\text{ (mW)}^{-1}$  into a LoD of  $22\text{ mg dl}^{-1}$ .

optical system is in order to quantitatively measure Raman scattering intensity in microfluidics.

In particular due to the careful sample exchange at the microfluidic inlet we can guarantee a stable optical alignment throughout a series of Raman measurements. We perform instantaneous blood plasma fractionation with the prepared whole blood samples of known free hemoglobin concentrations. Confocal Raman excitation is directed to the cell-free plasma domain in the microfluidic whole blood flow. As the free hemoglobin concentration of whole blood varies, the Raman intensity of the prominent hemoglobin peak at  $1375\text{ cm}^{-1}$  in Fig. 6(c) changes. The smoothed Raman peak intensity after the baseline correction scales monotonically with the hemoglobin concentration. Fig. 6(d) shows a linear scaling and high reproducibility at all analyzed concentrations. Each error bar results from three independent Raman measurements on dynamically fractionated blood plasma at the same hemoglobin concentration. Accordingly, free hemoglobin concentrations of unknown whole blood samples can be quantified. Fig. 6(c and d) show that there is a negligible offset in terms of free hemoglobin which is natural in any whole blood sample. It means, furthermore, that no *in vitro* hemolysis occurs during our microfluidic blood experiments.

Fig. 7 illustrates the applied baseline correction method and determines the level of detection (LoD) for free hemo-







In our first application example we demonstrate high sensitivity and appreciable linearity in free hemoglobin sensing by means of resonance Raman spectroscopy. The excitation wavelength has been chosen in order to specifically probe for hemoglobin. Fortunately, the Raman intensity from hemoglobin is strong enough to overcome the fluorescent background. This method enables instantaneous hemolysis detection on whole blood.

In our second application example we focus on the much weaker Raman scattering from microfluidic blood plasma domains at near-infrared excitation. The background Raman intensity from PDMS dominates over the plasma signal. However, the scaled subtraction of the background spectrum allows the restoration of the pure plasma signature. Mixtures of blood plasma with metallic colloids show enhanced Raman scattering compared to regular Raman spectra which we reproduce in the microfluidic setting. Such mixtures have potential for example in cancer diagnostics<sup>19,20</sup> and can be applied in microfluidics in order to reduce both colloid and blood consumption.

## Conflicts of interest

There are no conflicts of interest to declare.

## Acknowledgements

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