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Detection of Sodium and Potassium in Single Human Red Blood Cells by 193-nm Laser Ablative Sampling: A Feasibility Demonstration

C. W. Ng and N. H. Cheung*

Department of Physics, Hong Kong Baptist University, Waterloo Road, Kowloon, Hong Kong, People's Republic of China

The feasibility of quantifying sodium and potassium in single human erythrocytes was demonstrated by spectrochemical analysis of emissions from plasmas produced by 193-nm laser ablation of blood cells confined in a sheath flow. In one scheme, single blood cells that happened to be in the ablation volume were sampled. In another scheme, individual blood cells were first sighted and then synchronously ablated downstream. Plasma emission spectra of single ablated cells were captured, and the ratios of the analyte line intensity to the root-mean-square fluctuation of the continuum background were measured to be about 18 for sodium and 30 for potassium.

Increasingly more biomedical problems are both interesting and challenging to analytical scientists. One example is the manipulation and analysis of microspecimens, be they single biological cells or DNA's. Single-cell chemical analysis is needed for various clinical applications: early diagnosis of diseases such as AIDS or tumor metastasis, measurement of cell specificity in drug uptake, and monitoring of cell-to-cell variation of biochemicals, to name a few.¹ A specific problem is to quantify the metabolic electrolytes such as sodium and potassium in single human red blood cells (RBC's), at sampling rates rapid enough (~100 Hz) to yield meaningful statistics for clinical profiling. Each RBC is only about 8 μm in diameter and is no more than 2 μm thick, with typically 16 fg of sodium and 340 fg of potassium.² These quantities are orders of magnitude lower than the mass detection limits of conventional atomic emission or absorption spectrometry. Moreover, cell viability demands a technique that is compatible with water and the atmosphere. Flow cytometry and fluorescence microscopy are candidate techniques.^{3,4} They rely on tagging specific target molecules with fluorescent labels for detection. But quantitative accuracy may be sacrificed because tagging reactions may be incomplete and interference from other species, including the reagent, can be severe. Tagging specificity and spectral

bandwidths also preclude multicomponent analysis. Another promising technique is laser-induced breakdown spectroscopy (LIBS).⁵ We will show experimentally that a modified form of LIBS is a feasible approach.

In conventional LIBS, intense laser pulses of visible or IR wavelengths are used to ablate sample targets to produce luminous plasma plumes with characteristic emissions that help to determine the target compositions. However, the high temperature (a few electronvolts) of the plasma will cause extensive ionization of most elements.⁶ The analyte lines of neutral atoms will therefore be attenuated while the plasma continuum emissions due to the numerous free electrons will be overwhelming.⁷ The violent and explosive ablation will also cause instabilities in the plume, especially for liquid targets. As a result, the signal-to-noise (S/N) ratio is seriously compromised. Lowering the laser fluence will suppress the continuum emissions but the analyte signal will be attenuated even more.⁷ This is because a minimum irradiance is required to induce breakdown.

Interestingly, we demonstrated that luminous plasmas could be produced by ablating aqueous samples with ArF (193 nm) laser pulses at subbreakdown fluences.⁸ The plasma temperature was measured spectroscopically to be only about 0.5 eV, which is ideal for the excitation of analyte atoms such as sodium.⁷ The continuum background was shown to be relatively weak.⁷ Impressive mass detection limits (sub-picograms for Na) and broad dynamic ranges were demonstrated.⁸ The detection limits could be further reduced by shrinking the sampled volume with a modified sheath flow arrangement.⁹ With RBC's flowing along such a sheath flow, laser ablative sampling of multiple cells (about one to three cells per shot) for the determination of intracellular Na and K was attempted.⁹ The detection sensitivities (S/N ~ 2) for intracellular Na and K were estimated to be about 50 and 150 fg, respectively, to be compared with the typical cellular contents of 16 fg of Na and 340 fg of K. For single-cell analysis, further improvement in sensitivity is therefore needed. In what follows, we will show that the mass detectability could be improved by using finer sample jets and optical multichannel detection with nanosecond gating.

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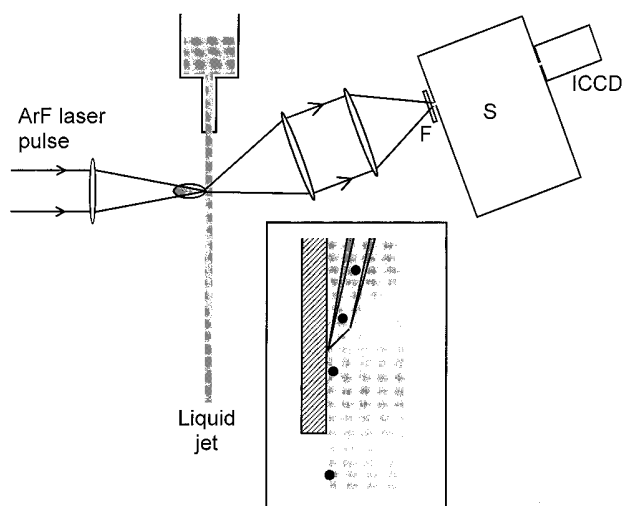


Figure 1. Schematic of the experimental setup for 193-nm laser ablative sampling of single human red blood cells in a sheath flow: F, UV-blocking filter; S, spectrograph; ICCD, intensified charge-coupled device array detector. Inset: magnified nozzle of the flow cell. The tapered capillary (50- μm i.d.) rests against the inside of the outer glass tubing (400- μm i.d.). Blood cells (8- μm diameter) are allowed to flow on the *outside* of the sheath in single file for eventual sampling downstream.

We will demonstrate that unambiguous single-cell spectra can be captured by synchronized ablation.

EXPERIMENTAL SECTION

The experimental setup is shown schematically in Figure 1. Details are given elsewhere.^{9,10} Briefly, centrifuge-washed RBC's were suspended in 8% glucose at 10 \times dilution and pressure-fed (7 psi) down a quartz capillary (Polymicro) of 50- μm i.d. and 140- μm o.d. at a flow rate of 4 $\mu\text{L}/\text{min}$. The capillary was slightly bent at a point about 8 mm above the tip. The bend forced the pointed tip to rest against the inside of a glass tubing (400- μm i.d.) through which the sheath liquid of deionized water was allowed to flow. This caused the blood sample to flow on the *outside* of the sheath; otherwise, all plasma emissions would be quenched. Relative to the sample, the sheath was allowed to flow at a much faster rate of 15 mL/min. As a result, the RBC fluid was dragged along to form a very fine jet by hydrodynamical focusing.¹¹ To ensure streamline flow, the capillary wall was etched to 5 μm thick at the slanted tip (Figure 1, inset). With practice and a well-formed capillary tip, a sample jet as fine as 8 μm could be made to flow stably on the outside of the sheath as the fluid exited the glass tubing. The RBC's were spaced in single file when they entered the sampling region downstream. The ArF ablation laser (Lumonics EX-748) delivered 15-ns pulses focused to about 80 $\mu\text{m} \times 700 \mu\text{m}$ ($h \times w$). The fluence was about 4 J/cm². Emissions from the plasma were imaged onto the 1-mm entrance slit of a 0.5-m spectrograph (ARC Spectro-Pro 500) equipped with a 600 line/mm grating. The overall spectral resolution was about 3 nm. An intensified CCD (Oriel 77193-5) gateable to nanosecond was mounted on the exit slit. To avoid 193-nm scatter, the axis of the collection optics was tilted 15° above the horizontal plane. Filters for blocking UV wavelengths were placed at the entrance slit of the spectrograph.

Delicate alignments were necessary to ensure accurate beam delivery and maximum signal collection. For that reason, at the

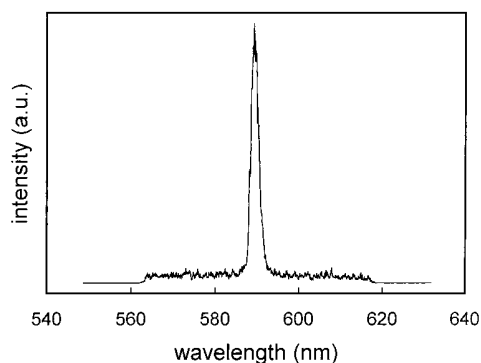


Figure 2. A typical plasma plume emission spectrum, produced by ArF laser ablation of an 8- μm -diameter sample jet. The sample solution contained 7 mM NaCl in 0.8 M HCl. The HCl acted as a strong absorbent at 193 nm. The laser fluence was about 4 J/cm². The spectrum corresponds to a single-shot event. The intensifier of the CCD was gated on for 1 μs . Time zero (firing of the ArF laser) was included. The spectral resolution was about 3 nm, and therefore the sodium 589.0 and 589.6-nm doublets were not resolved.

beginning of each data run before RBC's were sampled, a solution of 7 mM NaCl in 0.8 M HCl (as absorbent at 193 nm) was fed down the quartz capillary to be ablated. Optical beams and sample jets were then aligned to maximize the Na 589-nm signal emitted from the luminous plume. Once the alignment was completed, the flow cell was flushed and thoroughly cleaned with deionized water. The quartz capillary was then flushed twice with the blood solution before the RBC's were finally sampled.

At 10 \times dilution and with some sedimentation, there was only about one-fourth chance to have one RBC in the sampled volume on average¹² and the frequency of multiple cell ablation was negligible. Accordingly, any sodium or potassium spectra observed should correspond to signals from single RBC's. To suppress the weak but discernible continuum emissions that occurred initially, signals from the first 70 ns were gated off while the subsequent 1 μs of each event was integrated.

To be more certain of single-cell ablation, a synchronous sampling scheme was devised. RBC's were intercepted by a tightly focused He-Ne laser beam 200 μm above the ablation region. The forward Mie scatter signal was directed onto a photomultiplier tube. The photocurrent pulse, after suitable delay, triggered the ablation laser. To first ascertain the reliability of the synchronization, the photocurrent pulse was used to trigger a Nd:YAG laser (Continuum Surelite II) whose second harmonic output was employed to excite broad-band incoherent fluorescence of about 5-ns pulse width off rhodamine 6G dye for performing speckle-free flash photography. By varying the time delay, we could capture the sequence of "frozen" images of flowing RBC's and visualize the effects of sample flow rate.

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- (12) We imaged the blood cells with a microscope objective lens onto a high-speed CCD camera. The average spacing between two successive cells was about 300 μm . The 80- μm height of the laser spot size was about one-quarter of that spacing.

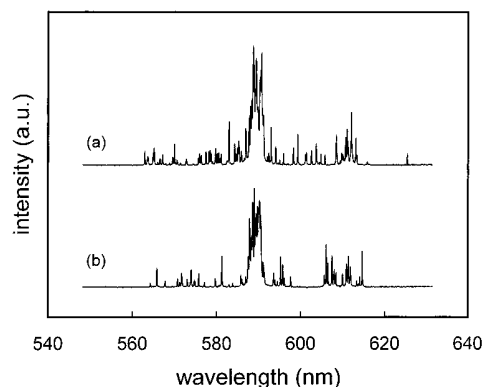


Figure 3. Typical plasma plume emission spectra produced by 193-nm laser ablative sampling of human red blood cells that had been centrifuge-washed and resuspended in 8% glucose. The laser fluence was about 4 J/cm². The initial 70 ns of each event was gated off, and the subsequent 1 μ s was integrated. Identical to the conditions of Figure 2, spectral resolution was about 3 nm, and therefore the sodium 589.0 and 589.6-nm doublets are not resolved. Both traces correspond to single-shot events when single blood cells were believed to have been sampled.

Synchronized RBC ablation was carried out by replacing the Nd:YAG laser with the ArF excimer laser. It should be pointed out, however, that RBC's suspended in glucose gave an unstable Mie signal, probably because of cell deformation.¹³ Only phosphate-buffered saline (PBS) as diluent could maintain cell shape and gave a stable Mie signal. Since PBS contained 150 mM sodium, strong background interference was likely. However, PBS contained only 4 mM potassium and hardly absorbed at 193 nm, so potassium emissions from RBC ablation should be minimally affected, as our data later showed. For that reason, only potassium emission spectra from synchronized RBC ablation were captured. To suppress the initial continuum emissions, the first 35 ns of each event was gated off while the subsequent 1 μ s was integrated. By suitable choice of the delay time between the Mie trigger and the ablation event, either blood cells or PBS solutions were ablated so that RBC versus blank spectra could be conveniently compared.

RESULTS AND DISCUSSION

As mentioned above, before RBC's were sampled, NaCl solutions were first ablated to aid alignment. Figure 2 shows a typical sodium spectrum produced by 193-nm ablation of 7 mM NaCl in 0.8 M HCl. It was a single-shot event. The integration time was 1 μ s, and $t = 0$ (firing of the ArF laser) was included. On the basis of the spectrum, the sensitivity of the technique can be roughly estimated as follows. The average, $\langle B \rangle$, as well as the standard deviation, σ , of the background intensity for a spectral region well away from the sodium peak can be computed. The background-subtracted signal, $\langle S \rangle$, is defined as the average intensity under the sodium peak minus $\langle B \rangle$. S/N , defined as $\langle S \rangle / \sigma$, can therefore be determined. For the spectrum shown in Figure 2, S/N is about 100. Given that the ablated volume, which was cylindrical in shape, was about 8 μ m in diameter and 80 μ m high, the amount of Na sampled was about 600 fg. Accordingly, the detectability ($S/N \sim 2$) was about 12 fg, which is comparable to the amount of Na found in a typical RBC. As we will show, the spectral background could be further suppressed if the weak but discernible continuum emissions during the initial few tens of

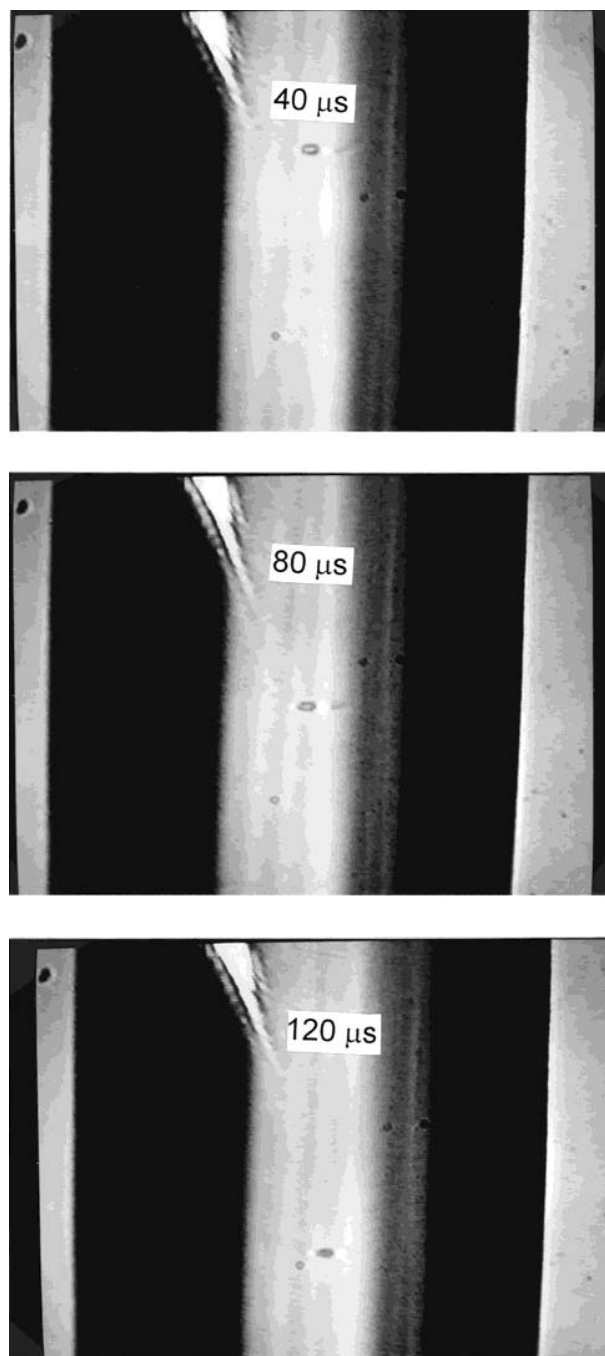


Figure 4. Synchronized flash photography to "freeze" flowing red blood cells. At flow rates of 4 μ L/min and 15 mL/min, respectively, for the blood sample and the sheath, a hydrodynamically focused stream of blood cells was made to flow in single-file fashion. Cells were intercepted upstream by a He-Ne laser beam. After a tunable time delay, Δt , the Mie scatter signal fired a Nd:YAG pulsed laser that induced fluorescence off a rhodamine 6G dye to illuminate the blood cells. The images of blood cells at time delays, Δt , of 40, 80, and 120 μ s are shown. The blood cells appeared elongated laterally because of the lens effect of the cylindrical liquid jet.

nanoseconds were gated off. The S/N could then be enhanced by about a factor of 6.

Figure 3 shows typical sodium spectra produced by nonsynchronous ablation of RBC's in glucose. As alluded to earlier,

(13) Reference 3, Chapter 5.

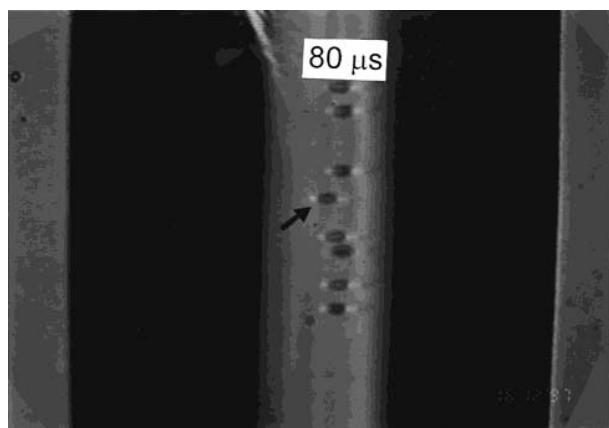


Figure 5. A flash photograph showing the effect of increased flow rate of the blood sample. The time delay was $80\ \mu\text{s}$. The sample flow rate was increased from the normal $4\ \mu\text{L}/\text{min}$ to about $70\ \mu\text{L}/\text{min}$ while the sheath flow rate was kept constant at $15\ \text{mL}/\text{min}$. In comparison with Figure 4 ($80\ \mu\text{s}$), the increased cell flux and the slight divergence of the sample stream are clearly observable. The constancy of the vertical position of the selected cell (marked with an arrow) is also apparent.

because there was only about one-fourth chance to have one RBC in the sampled volume, blank traces were seen most often and only occasionally were the unresolved Na 589.0- and 589.6-nm emissions observed (Figure 3). We believe these spectra correspond to signals from single RBC's.

Synchronized flash photographs of flowing RBC's are shown in Figure 4. Varying the time delay between the Mie trigger and the firing of the Nd:YAG laser allowed a sequence of "frozen" RBC's to be observed. The RBC's appeared elongated laterally because of the lens effect of the cylindrical liquid jet. The images demonstrated the accurate and reliable positioning of the RBC's. The cell location shown in Figure 4 ($120\ \mu\text{s}$) corresponds to the ablation position. No other RBC was observed within the ablation region, thus guaranteeing the ablation of only one cell at a time. The falling rate of $2\ \text{m s}^{-1}$, as gauged from Figure 4, was consistent with the measured sheath flow rate. Increasing the capillary flow rate beyond the normal operating range would cause divergence of the sample jet and an increase in the RBC flux, as Figure 5 clearly shows. It is interesting to note that even for such a case, one RBC could still be "frozen" (marked with an arrow in Figure 5) while other RBC's in the background wandered about from shot-to-shot. This demonstrated the selectivity of the synchronization scheme.

Typical potassium spectra produced by synchronous ablation of RBC's in PBS are shown in Figure 6. The time delay between the Mie trigger and the firing of the ArF laser was tuned on for cell ablation (Figure 6, top two traces) and off for blank traces (Figure 6, bottom trace).

To estimate the detection limits for intracellular K, we first computed the spectral S/N using the procedure mentioned previously. For the spectra shown in the top two traces of Figure 6, S/N was about 30. Strictly speaking, one should define detection limits in terms of reproducibility of results, which would call for the ablation of identical test cells. In our case, on the basis of the computed S/N, a rough order-of-magnitude estimate of the detection limit could be calculated. Assuming that the average

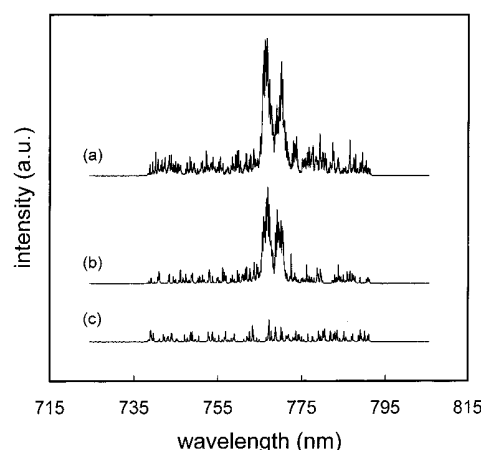


Figure 6. Typical plasma plume emission spectra produced by synchronous 193-nm laser ablative sampling of human red blood cells that had been centrifuge-washed and resuspended in phosphate-buffered saline. The laser fluence was about $4\ \text{J}/\text{cm}^2$. The initial 35 ns of each event was gated off, and the subsequent $1\ \mu\text{s}$ was integrated. The ArF laser was timed to fire at the blood cells for the top two traces, while it was timed to miss the blood cells for the bottom trace. Spectral resolution was about 3 nm, and the potassium 766.5 nm and 769.9-nm doublets are clearly resolved in the top two single-cell spectra.

cellular content of potassium is about 340 fg, the mass detection limit ($S/N \sim 2$) for potassium would be on the order of 40 fg. Analogously, on the basis of the sodium spectra shown in Figure 4, S/N was measured to be about 18. Assuming that the average amount of sodium per RBC is about 16 fg, the mass detection limit for intracellular sodium would be on the order of 2 fg.

CONCLUSIONS

We have demonstrated the feasibility of quantifying potassium and sodium in single RBC's by spectrochemical analysis of emissions from plasmas produced by 193-nm laser ablation of RBC's confined in a sheath flow. By using hydrodynamically focused jets of cellular dimensions and spectral detection based on gateable intensifiers and CCD arrays, we demonstrated impressive sensitivity that enabled single-cell analysis. A sight-and-fire experimental setup was configured for synchronous cell ablation. The ratios of the analyte signal to the root-mean-square fluctuation of the spectral background were measured to be about 30 for potassium and 18 for sodium, respectively, which were high enough for single-RBC analysis. Two key issues await further investigation: a reliable method to form the tip geometry of the capillary and a signal calibration protocol. Although clinical applications are not yet practical, it is worth noting that this analytical technique, being as simple and inexpensive as LIBS but more sensitive, is applicable to the elemental analysis of most aqueous samples and suspensions, be they live cells or otherwise.

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