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SHORT COMMUNICATION

Evaporative Preconcentration of Fluorescent Protein Samples in Capillary Based Microplates

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Abstract The preconcentration of analytes is important in biochemical analysis as it offers the ability to detect for trace species, and increase signal-to-noise ratios when using optical sensing on fluorophores. A strong advantage of the evaporation technique lies in its ability to operate without the need of any energy source; albeit major challenges exist on how to increase the surface area exposure to air for heightened evaporation, ensure no further increases once specified analyte concentrations have been achieved, and not needing any intervening membranes. We demonstrate here that the droplet creation and retraction approach in capillary based microplates offers such abilities whilst at the same time facilitating mixing.

Keywords Microplate · Capillary · Evaporation · Preconcentration · Green fluorescent protein

Introduction

The microplate is a standard tool in analytical research and clinical diagnostic testing laboratories. The trend in microplate instrumentation, in particular for high throughput screening (HTS), is for more effective ways to dispense and manage the testing of increasingly smaller liquid volumes. Miniaturized assays place greater demands on handling accuracy; and attempts to address this with more complex and precise machinery will translate to higher instrumentation costs. We have previously reported the idea of having the microplate wells themselves functioning like capillaries [1]. With this method, droplets can be released at the entrance of the well and will fill into it by capillary force alone if the well surface is hydrophilic.

Sample/reagent mixing is important in microplate instrumentation. The predominant approach currently adopted is for it to be done prior to dispensation into the microplate wells. When assays involve a mixture of different proportions of reagents, this approach can be a bottleneck in the process. A current major thrust is to accomplish mixing within the well itself. A variety of methods, ranging from the use of physical and magnetic agitation [2, 3], capillary concentration convection [4], and ultrasonic mixing [5, 6], have been reported for this on standard microplates. These approaches, however, cannot be directly translated for use in capillary well microplates. A method of creating strong localized mixing vortices by vibration offers some possibilities but is somewhat limited by the need for a T-junction channel [7].

A recent approach reported applying positive air pressure from one end of the capillary so that the liquid extends out from the opposite end to create a pendant drop (Fig. 1(a)) [8] from an original dispensed condition (Fig. 1(b)). As the drop forms, internal circulation occurs within as a result of the liquid along the axis center moving faster and making a return when it meets the slower front contact surface of the drop. Considerable mass transfer occurs as a consequence of this circulation gives rise to mixing. When

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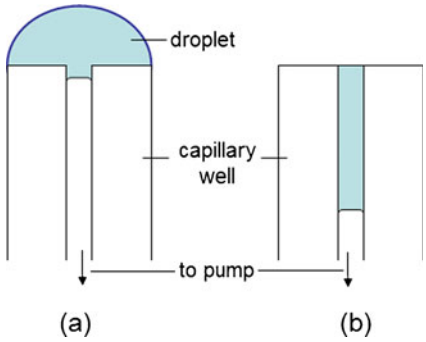

Fig. 1 Schematic illustration of pendant drop created (**a**) in a capillary from its original state (**b**) using air pressure delivery from the opposite end

the drop is drawn back into capillary, a similar circulation but in the reverse sense occurs to further facilitate mixing.

The preconcentration of analytes is acknowledged to be an important procedure in biochemical analysis, particularly when small liquid volumes are used. The advantages offered include the ability to detect for trace species, and an increase in signal-to-noise ratios when using optical sensing. Sample preconcentration methods can broadly be classified as surface binding (solid phase extraction [9]), electrokinetic equilibrium (isotachophoresis [10], isoelectric focusing [11], temperature gradient focusing [12]), and membrane based (electrophoretic filtering [13], evaporation [14], concentration polarization [15]). A strong advantage of the evaporation technique lies in its ability to operate without the need of any energy source. Major challenges with this technique however exist on how to increase the surface area exposure to air for maximal evaporation and to ensure no further increases once specified analyte concentrations have been achieved. It will also benefit from not needing any intervening membranes as residues of the analyte (which may be expensive) can be trapped there. We demonstrate here that the droplet creation and retraction approach erstwhile reported [8] offers such abilities.

Experimental

The experimental sample used was enhanced green fluorescent protein (EGFP), isolated from genetically modified Escherichia coli (E. coli) and purified by chromatography. After elution of the proteins from the chromatographic matrix, the sample was then dialysed into Tris-Cl pH 8.0, checked for purity by SDS-PAGE and quantified using the BCA protein assay (Pierce, USA). A fixed volume of 2 μl of the analyte was delivered into capillary wells of internal diameter 0.62 mm using a manual pipetter (model: Eppendorf Research® pipettes, 0.1–2.5 μl). The ambient condition was controlled by placing the samples in an environmental cabinet (Thermoline Scientific TRH-150) in which the temperature and humidity was monitored. Two sets of data were collected; one in which the analyte remained in the capillary and the other with the analyte pushed out to form a pendant drop at the tip. The latter was accomplished using a flexible tube connecting the capillary well at the opposite end to a syringe pump (Adelab Scientific NE-1000). Using a push and pull operation generated at the syringe pump, it was possible to apply positive and negative air pressure to create and diminish the pendant drop respectively. The temperature of the environmental cabinet was kept at 26 °C while the humidity was varied. Previously, enhanced green fluorescent protein has been shown to be highly stable at temperatures below 70 °C [16]. This was adopted as high temperatures may result in rapid deterioration of the protein. When taken out from the environmental chamber, the analyte in capillaries was measured for the volume remaining in the capillary as well as concentration. The images of the analytes were recorded using a coupled fluorescence, luminescence and radio-isotope scanner (Typhoon Trio from Amershan Bioscience). The volume and concentration were then analyzed from the scanned images using the ImageQuant analysis software.

Results and Discussion

Figure 2 furnishes an example fluorescence image obtained with capillaries without (a) and with (b) the scheme of droplet evaporation. The progression of time in which the samples were subjected to in the environmental chamber is from left to right. It can be seen that the onset of droplet evaporation with time (b) results in the volume decreasing and the concentration increasing (manifested as brighter fluorescence). The volume and concentration

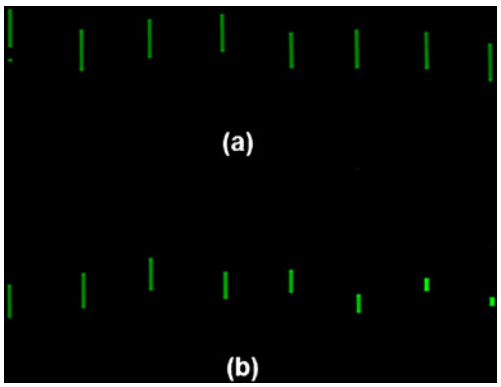

Fig. 2 Example fluorescence image obtained with capillaries without (**a**) and with (**b**) the scheme of droplet evaporation. The progression of time in which the samples were subjected to in the environmental chamber is from left to right

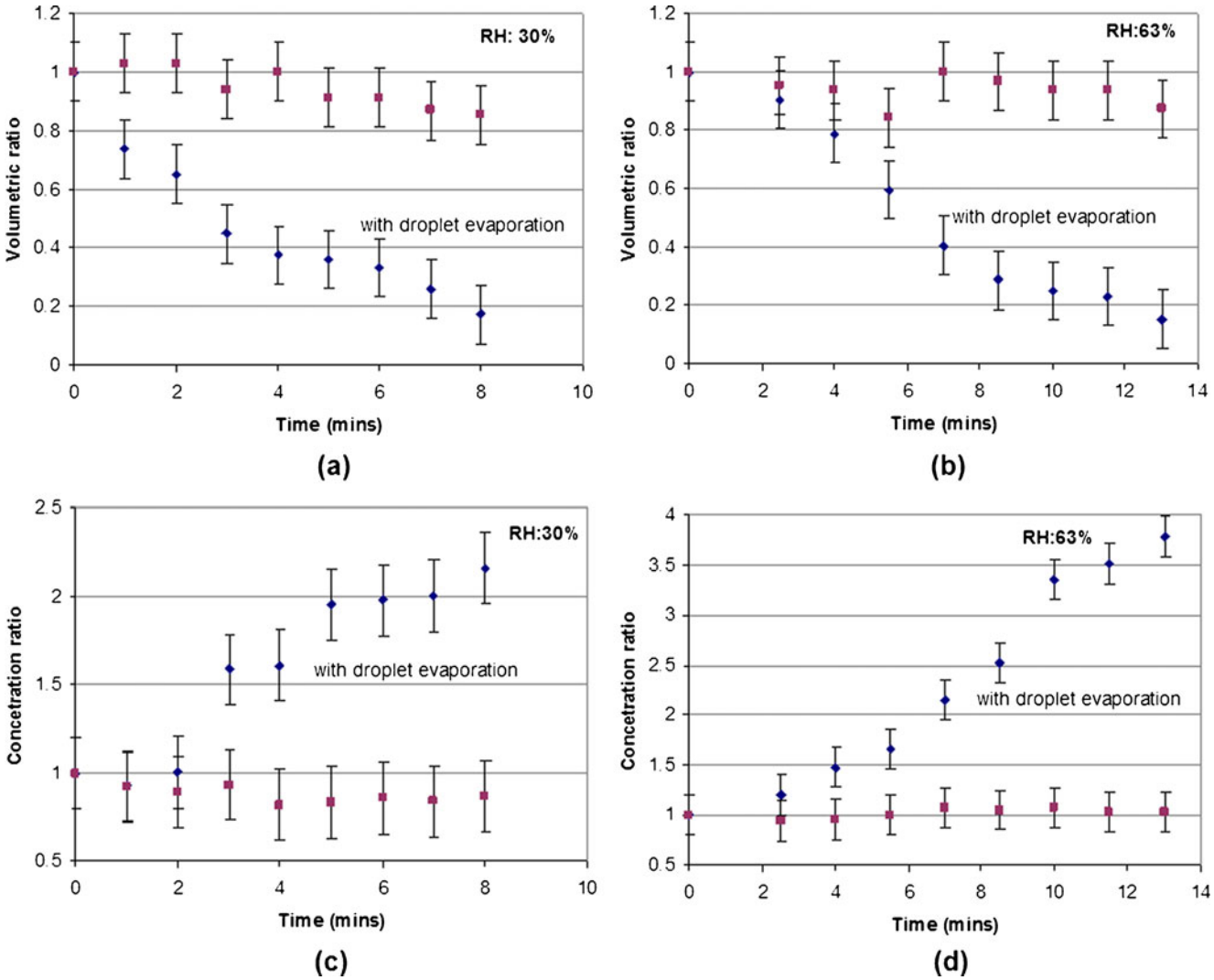

Fig. 3 Graphs of volumetric ratio change with time at 30% (**a**) and 63% (**b**) averaged relative humidity with and without the droplet evaporation scheme. Also presented are graphs of concentration ratio change with time at 30% (**a**) and 63% (**b**) averaged relative humidity with and without the droplet evaporation scheme

were respectively evaluated in a non-dimensional sense using

$$\text{volumetric ratio} = \text{volume}/\text{initial volume} \quad (1)$$

$$\text{concentration ratio} = \text{concentration}/\text{initial concentration} \quad (2)$$

Graphs of volumetric ratio change with time at 30% (a) and 63% (b) averaged relative humidity with and without the droplet evaporation scheme are presented in Figs. 3(a) and (b) respectively. In both cases, it is clear that the introduction of a droplet results in significant reduction in volume; indicating enhanced evaporation. Figures 3(c) and (d) show graphs of concentration ratio change with time at 30% and 63% averaged relative humidity with and without the droplet evaporation scheme respectively. As expected, an increase in concentration results from the enhanced evaporation arising from the formed droplet. If a 2 times increase in original concentration is used as benchmark, the data in Table 1 (column 2) indicates a much faster

Table 1 Tabulation of results from the evaporative preconcentration process in which the average relative humidity was varied.

Average Relative Humidity (%)	Time to 2X concentration of the original (minutes)	Highest concentration achieved (times of the original)	Time to highest concentration achieved (minutes)
30	6.5	2.16	8
50	7	3.25	10.5
63	7.5	3.78	11.5
70	12	3.80	16
85	35	5.10	120

attainment with low relative humidity (e.g. 6.5 minutes at 30% RH as opposed to 35 minutes at 80% RH). This is an expected result naturally. We have set a sample volume of 0.1 times the original as the highest that we can measure concentration from in each case. It turns out that higher concentrations could be attained with higher relative humidity (column 3 of Table 1); albeit over a longer period of time. It appeared that the protein has undergone denaturation leading to some loss in fluorescence capability when the subject to higher evaporation rates (i.e. low humidity). This may be caused by rapid evaporation with localized cooling at the surface of the pendant droplet as water escaping into the gaseous phase may result in a relatively larger surface area of liquid-vapor interface formed. Entrapment of the proteins within this transition layer may expose them to greater denaturation forces at the air-water interface [17] akin to that observed for freeze denaturation of proteins in the ice-liquid interface [18]. Furthermore, it has been demonstrated that addition of a surfactant to compete with recombinant Factor XIII (rFXIII) at the air-water interface was useful in reducing agitation-induced aggregation of the protein [19]. The preferential positioning of the surfactant relative to (rFXIII) at the air-water interface accorded protection of rFXIII to structural perturbation and aggregation is evidenced by retention of its intrinsic fluorescence property. In view of this, such behavior may also be present in other modes of preconcentration. An important finding is that both the volume and concentration of sample can be kept constant in the capillary if the pendant droplet is not created.

In applying this approach, it is necessary to ensure that liquid from the capillary is pushed sufficiently out to form the pendant drop. In the same vein, care has to be taken not to cause the pendant drop to detach to result in loss of the sample. After some practice, we found that such a control was not difficult to accomplish. It should be noted that the manner of evaporation from the droplet is determined from its shape [20, 21]. It has been recently determined that the surface plays a role in altering the droplet shape and thus the rate of evaporation [22]. The proposed preconcentration scheme will therefore benefit from keeping this factor constant. Apart from the ability to mix [8], the concentration and volume can also be monitored in-situ using an optical side access approach for the capillary well microplates scheme [23, 24]. Overall, this helps to make the approach much more attractive over standard microplates.

Conclusions

In summary, we demonstrated here the droplet creation and retraction approach in capillary based microplates offers the ability to attain preconcentration of fluorescence samples whilst at the same time facilitating mixing. The preconcentration rate is faster with lower humidity; albeit higher concentrations can be achieved using higher humidity. The approach is simple and robust and serves to underline another added advantage of capillary wells over standard microplates.

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