

Micro sequential injection: fermentation monitoring of ammonia, glycerol, glucose, and free iron using the novel lab-on-valve system

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Using an integrated lab-on-valve manifold in a microfluidic sequential injection format (μ SI), automated sample processing has been developed for off-line and on-line monitoring of small-scale fermentations. Spectrophotometric assays of ammonia, glucose, glycerol, and free iron were downscaled to use micro-quantities of commercial reagents. By monitoring the reaction rate, the response curves in a stopped-flow mode generate linear calibration curves for ammonia [$r^2 = 1.000$ (0.9% SE)], glycerol [$r^2 = 0.999$ (1.1% SE)], glucose [$r^2 = 0.999$ (1.1% SE)], and free iron [$r^2 = 0.999$ (1.5% SE)]. Since sample dilution and reagent quantities are easily adjusted within the programmable SI format, the lab-on-valve system can accommodate samples over a wide concentration range (ammonia: 3–1200 ppm; glycerol: 20–120 ppm; glucose: 35–1000 ppm; and free iron: 80–400 ppm). This work demonstrates the key advantages of miniaturization through the reduction of sample and reagent use, minimizing waste and providing a compact yet reliable instrument. The lab-on-valve manifold uses a universal hardware configuration for all analyses, only requiring changes in software protocol and choice of reagents. All of these features are of particular importance to small-scale experimental fermentation where multiple analyte analyses are needed in real-time using small sample volumes. It is hoped that this first real-life application of the lab-on-valve manifold will serve not only as a model system to downscale assays in a practical fashion, but will also inspire and promote the use of the integrated μ SI manifold approach for a wider range of biotechnological applications.

Introduction

Miniaturization of flow based assays is at present an actively pursued topic in analytical research. While much of this effort is based on technology that is focused on the implementation of silicon microchips (MEMS or μ TAS) where nanoliter to picoliter quantities of sample are being used,¹ less attention is being paid to the needs of real-life applications. MEMS technology has several serious caveats to its immediate use in a practical sense. Reliability and robustness of operation is a problem where micro-channels are prone to clog or experience severe surface fouling. Interfacing these micro-devices to needed support electronics and hardware often result in an overall package that is not altogether compact. Finally minimizing sample/reagent consumption to the sub-microliter range is often not a critical issue. Indeed, it could be very much problematic in that micro sampling can introduce sampling errors, analysis errors through evaporation, and operational errors due to precipitation of solids. In many areas, simply providing a compact and robust instrument that down scales sample and reagent use to a few microliters would be of great benefit. The case in point is in the monitoring of a research fermentor, which is carried out in a small-scale and requires minimal sample withdrawal over the course of the process. Importantly, fermentation solutions are suspensions of cells and protein matter that could easily clog flow channels on a microfabricated scale. These considerations have lead toward a more practical level of miniaturization through the design of an integrated sequential injection manifold known as the lab-on-valve.^{2,3}

Conventional flow injection (FI) is a well-established technique for quick and precise off-line measurements of a

variety of components important to fermentation research.^{4,5} With its proven reliability and versatility, FI is used throughout the biotechnology industry for process monitoring (Table 1). Sequential injection⁶ (SI), the second generation of FIA, was introduced to further reduce reagent consumption and to make the technique more compatible with computer automation. While 'classical' FI protocol necessitates the physical reconfiguration of the flow manifold to perform different assays, SI methodology allows for all experimental manipulations to be altered by software control.^{2,3,6}

Based on the principles of sequential injection, the lab-on-valve manifold (Fig. 1a) use a central processing unit that integrates all flow channels and sampling ports within a single multipurpose detector cell^{2,3} (Fig. 1b and c). Different reagent based assays can be performed using the same base structure since variations in experimental protocols (reagent additions, dilution, mixing, and incubation time) are programmable. The important feature of the μ SI design is in the precise fabrication of very short channels between sample/reagent ports and the detector. Precision fabrication of this monolithic structure produces smooth conduit surfaces that experience minimal surface contamination and thus is well suited for bioassays or suspended matter. The use of flow reversal in this μ SI approach provides all of the needed reagent/sample mixing while minimizing sample travel and dispersion.^{2,3} Reagent based assays must rely on highly repeatable sample/reagent handling and precise control of incubation periods. The latter parameter is critical if the reaction on which the assay is based does not come to equilibrium prior to detection. Computer activated stopped-flow can select a precise segment of the sample/reagent zone that is reacting within the detector after initially being assembled and mixed in the holding coil. By precisely stopping

at the desired segment within the flow through cell, the reaction rate can be easily monitored.^{2,3} This approach has been used in this work to develop methods for the determination of ammonia,⁷ glycerol,^{8,9} glucose,¹⁰ and free iron¹¹ ion in fermentation samples. The overall experimental execution, as detailed through a typical programming protocol (Table 2), can serve as a guide for adaptation to other reagent based assays using the same μ SI hardware.

Experimental

Instrument

The sequential injection system (FIALab-3000, FIALab Instruments, Medina, WA, USA, <http://www.flowinjection.com>) consists of a syringe pump (500 μ l volume) driven by a stepper motor (24 000-step full range), a six-port selector valve, and a single speed unidirectional peristaltic minipump (Fig. 1a). Tungsten-Halogen lamp (LS-1, Ocean Optics Inc., El Dorado Hills, CA, USA, <http://www.oceanoptics.com>) was used as the VIS light source for a UV-VIS spectrophotometer (S2000, Ocean Optics Inc., El Dorado Hills, CA, USA, <http://www.oceanoptics.com>). An AIS UV-2D deuterium lamp (Analytical Instrument Systems Inc., Flemington, NJ, USA, <http://www.aishome.com>) was used as the UV source. Fiber optic cables, furnished with a stainless steel tip (400 micron, tip 0.0625" OD) were used to connect the flow cell to the light source and the spectrophotometer. Data was collected by FIALab for Windows (version 5.7.8) installed on a PC (Pentium II 300 MHz, 64 MB RAM) with Windows 98 (Microsoft Inc., Edmond, WA, USA) operating system.

The lab-on-valve manifold was fabricated in-house and interfaced to match a six-port selector valve as described earlier.^{2,3} The central inlet was connected to a holding coil. The flow cell was designed to connect port #2 of the six-port valve to the integrated flow-through detector. The sampling port was set to port #5 while the waste was designated to #1 and all remaining ports were assigned to reagents and carrier (spacer) solutions. The flow cell was configured for absorbance measurements as shown in Fig. 1a. Note that the top of the fiber optic cable connected to the light source is inserted into the channel allowing adjustment of the flow cell path length (Fig. 1b). In this way, the tips of both optical fibers defined the detector's flow cell volume (20 μ L at 10 mm). For assays with high analyte concentration that formed an intense colored product, the flow cell was configured to a shorter optical path (1 mm) with low internal volume (2 μ L) (Fig. 1c).

Fermentor seed culture

E. coli W3110 (ATCC 27325) was streaked out onto plates of LB agar (Difco) and grown overnight at 30 °C. An isolated

colony was picked and used to inoculate a 2.0 L baffled shake flask containing 200 mL of Super Broth II (Becton Dickenson) with 5 g L⁻¹ glycerol added. This flask was grown 20 h at 30 °C, in a shaking incubator with agitation set at 250 rpm.

Fermentor culture

A 20 L BioFlo 4500 fermentor (New Brunswick Scientific, Edison, NJ, USA, <http://www.nbsc.com>) was used for the experiment. The fermentor was fitted with a 19 mm Flownamics FISP *in situ* sampling probe (Flownamics Instruments Inc, Madison, WI, USA). The fermentation recipe contained the following components: (NH₄)₂SO₄, 1.0 g L⁻¹; KH₂PO₄, 0.85 g L⁻¹; K₂HPO₄, 5.70 g L⁻¹, Yeast Extract (Difco) 5.0 g L⁻¹, antifoam 289 (Sigma) 0.1 ml L⁻¹. The fermentor was filled to 15 L with doubly ionized (DI) H₂O and sterilized in place. After cool down, 125 mL of a 60% glucose solution (m/v%) was added to the fermentor along with 125 mL of 1 M MgSO₄. The starting pH was adjusted to pH 7.3 by addition of 2 M NaOH. The fermentation was run at 32 °C with aeration set at 1 vvm and initial agitation set at 300 rpm. Dissolved oxygen (DO) was controlled not to drop below 30% saturation using a DO control loop linked to agitation speed (ML6100, New Brunswick Scientific, Edison, NJ, USA). No pH control was used. The fermentation was inoculated with 200 ml of the shake flask culture ($A_{600} = 6.55$). The fermentation was run for 6.5 h with the A_{600} rising from an initial 0.22 to a final of 3.22.

Reagents and standards

Ammonia assay. Ammonia reagent powder pillow (Hach Co., Loveland, CO, USA, <http://www.hach.com>) used for this assay composes reagents for the well-known Berthelot's assay.⁷ It contains lithium hydride, sodium citrate, sodium nitroferrocyanide, sodium salicylate, and sodium tartrate. One powder pillow (approximately 0.9885 g) was dissolved in 5.0 mL water (double concentration, compared to manufacturer instruction). Diluting (1 + 1 v/v) a generic household bleach containing 5.25% (w/w) sodium hypochlorite with DI water made the stock hypochlorite solution. A 3000 ppm ammonia stock solution was made by dissolving 2.915 g ammonia sulfate, (NH₄)₂SO₄ (J. T. Baker Inc., Phillipsburg, NJ, USA, <http://www.jtbaker.com>) in 250 mL DI water. Other standards were made by serial dilutions from this stock solution. The carrier solution was made by diluting (1 + 99 v/v) a commercial detergent (Joy, Procter & Gamble, Cincinnati, OH, USA) with DI water. All reagents and standards were freshly prepared and degassed before used.

Glycerol assay. Triglyceride (GPO-Trinder) reagent A (337-40A, Sigma Diagnostics Inc., St. Louis, MO, USA, <http://www.sigma-aldrich.com>) was used for this assay. For the glycerol assay, only reagent A of the above kit was needed. The

Table 1 FI/SI techniques for monitoring fermentation substrates

Method	Analyte	Matrix	Reference ^a
FI	Sugar, lactic acid, protein, biomass glucose, lactate	Lactic acid cultivation mixture	4
		<i>Lactococcus cremoris</i> FD 1, <i>Escherichia coli</i> MT 102, and <i>Saccharomyces cerevisiae</i> DGI 342 cultivation mixture	5
	Ethanol	Wine fermentation mixture	12
	Glucose, lactate Glucose, L-lactic acid	Mammalian, <i>E. Coli</i> , and bacterial cell lines cultivation fermentation broths <i>Lactococcus lactis</i> ATCC 19435 cell line cultivation fermentation mixture	13 14
SI	D-lactic acid	<i>Lactobacillus delbrueckii</i> ATCC 9649 cell line cultivation fermentation broth	15
	Glucose, penicillin	<i>Penicillium chrysogenum</i> cultivation fermentation mixture	16
	Glucose, lactic acid, penicillin	Penicillin cultivation fermentation mixture	17
	Biomass of yeast cells	Yeast fermentation mixture	18

^a Note: Additional 50 references can be located in the database at <http://www.flowinjection.com>

triglyceride (GPO-Trinder) reagent A consists of adenosine-5'-triphosphate (ATP, 0.375 mmol L⁻¹), magnesium salt (3.75 mmol L⁻¹), 4-aminoantipyrine (4-AAP, 0.188 mmol L⁻¹), sodium-*N*-ethyl-*N*-(3-sulfo-propyl) *m*-anisidine (ESPA, 2.11 mmol L⁻¹), glycerol kinase (GK, 1250 U L⁻¹), glycerol phosphate oxidase (GPO, 2500 U L⁻¹), peroxidase (horse-radish, 2500 U L⁻¹), buffer (pH 7.0 at 25 °C), and sodium azide (0.02% w/w) as a preservative. The dry reagent mixture was reconstituted in 20.0 mL of DI water and kept in a sealed bottle. This reagent will remain stable for 60 d if refrigerated at 2–8 °C. Prior to use this reagent is allowed to warm to room temperature (25 °C). Glycerol (glycerol anhydride, J. T. Baker Inc.) standards were obtained from a serial dilution of a 5000 ppm glycerol stock in DI water. Carrier solution is the same as that of the ammonia assay.

Glucose assay. Infinity™ glucose reagent (18-20, Sigma) was used. It consists of adenosine-5'-triphosphate (ATP, 2.1 mmol L⁻¹), hexokinase (HK, >1500 U L⁻¹), glucose-6-phosphate dehydrogenase (G-6-PDH, >3200 U L⁻¹), buffer (pH 7 at 25 °C), and sodium azide (0.02% w/w) as a preservative. The dry reagent is reconstituted in 4.0 mL of DI water. This reagent is stable in a refrigerator at 2–8 °C and for up to 12 months. The reagent was warmed to room temperature (25 °C) prior to use. Glucose standards were made by a serial dilution from 5000 ppm D-glucose stock solution. All reagents and standards were freshly prepared and degassed. Carrier solution is the same as that of the ammonia assay.

Free iron assay. The iron assay kit (565-A, Sigma) was used. This assay kit includes four solutions: iron buffer reagent, UIBC buffer reagent, iron color reagent, and an iron standard. The iron buffer reagent contains hydroxylamine hydrochloride (1.5% w/v) in acetate buffer (pH 4.5) with an added surfactant. The iron

color reagent contains ferrozine (0.85% w/v) in hydroxylamine hydrochloride solution with added stabilizer. The iron standard solution (500 µg dL⁻¹) was used for calibration. Carrier used was degassed DI water with surfactant added.

Results and discussion

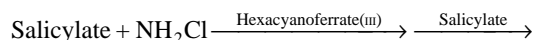
µSI protocol

The µSI technique allows sample and reagent solutions to be selected, mixed and diluted automatically. This is achieved by sequentially aspirating from the multi-port valve sample and reagent(s) into stacked zones into a holding coil (Fig. 1a). By reversing the flow direction the stacked zones disperse and begin to react in sequence as they are transported into the flow through detector cell. It has been shown^{3,6} that it is preferable to inject the sample zone first, followed by the reagent zone(s) in the sequence required by the chemistry. To promote good sample/reagent zone overlap and mixing, an additional spacer zone of carrier solution is aspirated into the holding coil. This spacer zone of carrier solution is ultimately aspirated into the holding coil, will increase the distance traveled and will increase dispersion within the sample/reagent zone as it is displaced further upstream into the holding coil. Next, flow reversal further promotes mutual mixing of sample with reagents as they are transported by flow reversal into the detector cell. The stopped flow timing must be carefully chosen to select a desired portion of the stacked zone within the flow cell for reaction rate monitoring. In summary, an overall experimental sequence comprises of four steps: (1) stacking of the sample, reagent(s) and spacer into the holding coil; (2) flow reversal toward flow detector cell; (3) stopped flow capture of a selected portion for measurement; and (4) washout of the conduit channel and flow through detector cell for the next assay cycle. Thus, when not in use, the channels and the flow cell are filled with carrier stream.

For high analyte concentrations, automated sample dilution is desired in order to obtain a readout within the calibration range. Since batch fermentations often start at high analyte concentrations, which are consumed during the fermentation process to a much lower level, a large dynamic working range for many of these analytes is needed. Dilution is achieved by inserting a large volume of carrier serving as a spacer following the sample aspiration. Next, most of this composite analyte/spacer zone is discarded into waste leaving a diluted portion at the tail of sample zone to be mixed with the reagents at the start of the assay cycle in a way discussed above.

The key to performing stopped flow kinetic measurements is to adjust the timing and transport of analyte/reagent zone so that the formation of the product is predominantly taking place within the flow cell. Fast reactions on the other hand are best monitored using a continuous flow mode, so that formation of the product is recorded as a transient peak when the composite zone is passing through the flow cell. Note that the assays for ammonia, glucose and glycerol described below are stopped flow kinetic assays, while the free iron assay is a continuous flow measurement of peak height.

Ammonia protocol



Indosalicylate (Indophenol dye)

Berthelot's method⁷ is a two-step reaction sequence wherein the first step ammonia reacts with hypochlorite to form the

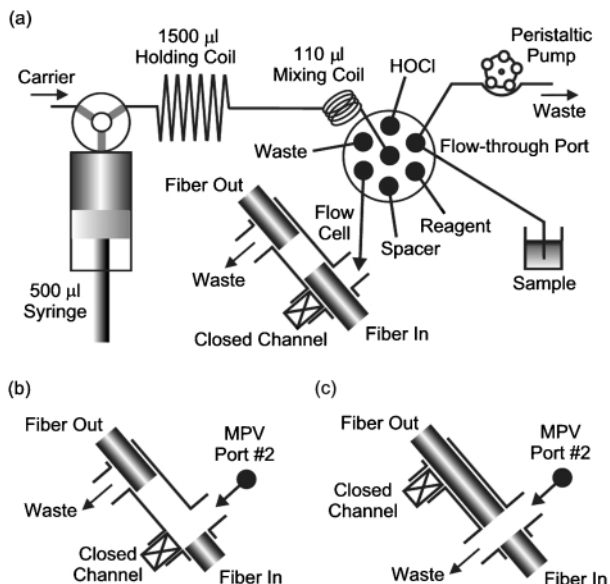


Fig. 1 (a) Schematic layout of the µSI instrument with the precision syringe pump (500 µL at 24 000 steps full stroke) connected to the holding coil and the mixing coil where the stacked zones of reagent/sample are made. The lab-on-valve device was mounted atop a six-way, multi-port valve, which allows for the fluidic operations of sample (port #5) and reagents (port #4 and 6) selections as well as the selection of the flow cell (port #2). An auxiliary peristaltic pump serves the flow-through port (port #5) for rapid sample exchange. The flow cell is shown in absorbance configuration using two optical fibers facing each other (3.0 mm light path). Inserting a rod with its od equal to channel id makes the closed channel in the lab-on-valve device. For further details, see text. (b) Flow cell configuration for longer optical path length (*i.e.* low absorbance measurements). (c) Flow cell configured for shorter optical path length (*i.e.* high absorbance measurements).

monochloroamine (NH₂Cl) product. Subsequently the monochloroamine reacts with phenol in an alkaline environment to form an Indophenol dye. Indophenol dissociates into blue Indophenol anion at high pH and is measured at 705 nm. When measuring the reaction rate in a μ SI mode, doubling the reagent concentration can substantially reduce the assay time. The μ SI method starts by stacking zones into the holding coil in the following order: sample, hypochlorite and the Hach reagent

followed by a carrier spacer (Fig. 2a). Following flow reversal, the composite zone was pushed into the detector cell for reaction rate measurement. Depending on the volume of displacement differences, concentration dependent rate curves are obtained. A displacement volume of 70 μ L was selected for the calibration curve since it yielded the steepest rate slopes. This established the timeline of the experimental protocol and allowed calibration of the system within a working range of

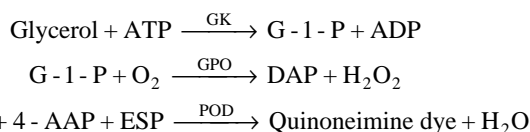
Table 2 μ SIA protocol^a in FIALab™. General μ SI programming sequence used for the glycerol analyses. All fluidic manipulations can be optimized by software control allowing the researcher to use the same universal hardware platform for a variety of analytes

Protocol	Note	Protocol	Note
Syringe Pump Command (?) K0R	Turn off backlash compensation of syringe pump.	Syringe Pump Flowrate (μ L s ⁻¹) 80	In-line dilution
Variable Define New DilutionDispense	DilutionDispense:	Syringe Pump Valve In	subroutine ^b for
DilutionDispense = 180	volume that need	Syringe Pump Aspirate (μ L) 200	high concentrate
Variable Define New SpacerVolume	to be dispensed in	Syringe Pump Delay Until Done	sample. Higher
SpacerVolume = 50	the final step of	Syringe Pump Valve Out	flow rate was
Variable Define New MeasurementPosition	in-line dilution.	Syringe Pump Flowrate (μ L s ⁻¹) 30	used in order to
MeasurementPosition = 70	ReactionTime:	Multiposition Valve Sample	have the
Variable Define New MeasurementDelay	Set to zero for the	Syringe Pump Aspirate (μ L) 30	maximum
MeasurementDelay = 55	measurement of	Syringe Pump Delay Until Done	dispersion along
Variable Define New ReactionTime	initial rate.	Multiposition Valve Carrier	the flow channel.
ReactionTime = 0	SettleDownTime:	Delay (s) 0.3	When 180 μ L was
Variable Define New SettleDownTime	minimize the	Syringe Pump Aspirate (μ L) 80	dispensed, the
SettleDownTime = 3	“micro-lens	Syringe Pump Delay Until Done	dilution factor
Variable Define New NumberOfRuns	effect” at the	Multiposition Valve Waste	was about 34. If
NumberOfRuns = 3	early stage of	Delay (s) 0.3	larger factor was
	detection.	Syringe Pump Dispense (μ L) 180	needed, dispense
		Syringe Pump Delay Until Done	more.
PeristalticPump On	Minimize sample	Multiposition Valve Reagent	Solution profile
Delay (s) 10	carry-over when	Syringe Pump Aspirate (μ L) 30	sequencing
PeristalticPump Off	changing samples.	Syringe Pump Delay Until Done	subroutine. ^c
		Delay (s) ReactionTime	Positioning the
		Multiposition Valve Carrier	measurement
		Syringe Pump Aspirate (μ L)	point in solution
		SpacerVolume	profile is critical
		Syringe Pump Delay Until Done	since it controls
		Multiposition Valve Flow Cell	the sensitivity of
		Syringe Pump Flowrate (μ L s ⁻¹) 30	the assay.
		Syringe Pump Dispense (μ L)	
		MeasurementPosition	
		Syringe Pump Delay Until Done	
		Delay (s) SettleDownTime	
Loop Start (#) NumberOfRuns	Repeat the same	Spectrometer Reference Scan	Start the ref. scan
	assay three times.	Spectrometer Absorbance Scanning	right before the
		Delay (s) MeasurementDelay	abs. scan in order
		Spectrometer Stop Scanning	to have the data
PeristalticPump On	Cleaning and		curve rises at the
Delay (s) 5	reagent prep		baseline.
PeristalticPump Off	subroutine: In		
Syringe Pump Flowrate (μ L s ⁻¹) 80	order to ensure the	Syringe Pump Flowrate (μ L s ⁻¹) 80	Finalization for a
Syringe Pump Valve In	freshness of	Syringe Pump Empty	single run in each
Syringe Pump Aspirate (μ L) 300	sample, a short	Syringe Pump Delay Until Done	sample. Using
Syringe Pump Delay Until Done	flush on flow-	Syringe Pump Valve In	high flow rate and
Multiposition Valve Flow Cell	through port was	Syringe Pump Aspirate (μ L) 300	flushing with
Syringe Pump Valve Out	used at the	Syringe Pump Delay Until Done	additional carrier
Syringe Pump Empty	beginning of each	Syringe Pump Valve Out	solution for
Syringe Pump Delay Until Done	run. Channels on	Syringe Pump Empty	restoring the
Multiposition Valve Carrier	MPV were	Syringe Pump Delay Until Done	initial condition
Syringe Pump Aspirate (μ L) 100	flushed with their		of the multi-
Syringe Pump Delay Until Done	individual reagent		purpose flow cell.
Multiposition Valve Reagent	before each run.		
Syringe Pump Aspirate (μ L) 20	Dead volume in	Loop End	
Syringe Pump Delay Until Done	the flow-through		
Multiposition Valve Sample	port was also		
Syringe Pump Aspirate (μ L) 30	considered.		
Syringe Pump Delay Until Done			
Multiposition Valve Waste			
Delay (s) 0.3			
Syringe Pump Empty			
Syringe Pump Delay Until Done			

^a Actual experimental protocol used in the glycerol assay. ^b In-line dilution subroutine could be ignored only if the concentration of raw sample falls into the dynamic range of the reagent(s). ^c If two-reagent assay system was used (e.g. ammonia assay, etc.), additional reagent aspiration steps should be added.

0–3000 ppm NH₃. Repeatability of the calibration is shown in Fig. 2b based on three replicate runs (blank to 1200 ppm NH₃).

Glycerol protocol



This assay is a two-step enzymatic reaction of which the final product, Quinoneimine dye, is detected at 540 nm.^{8,9} The concentration of dissolved oxygen is a limiting factor. Therefore, glycerol sample had to be prediluted prior to its addition to the enzymatic reagents. This was accomplished by aspirating 30 μL of glycerol into the holding coil, followed by 180 μL of carrier. The valve was then switched towards waste and 180 μL was expelled into waste (Table 2, dilution subroutine). This left approximately 30 μL of diluted glycerol in the holding coil, ready to be stacked with the multicomponent enzymatic reagent (30 μL) and carrier spacer (50 μL). Following flow reversal, the composite zone was displaced through the valve into the flow cell and held there for reaction rate measurement (Fig. 3a). A displacement volume of 70 μL provided optimized positioning in the flow cell for all reaction rate measurements in the 0–120 ppm glycerol ranges. Although the eventual depletion of oxygen affected the latter portion of the reaction rate, the initial reaction rate was all that was needed to yield a linear response

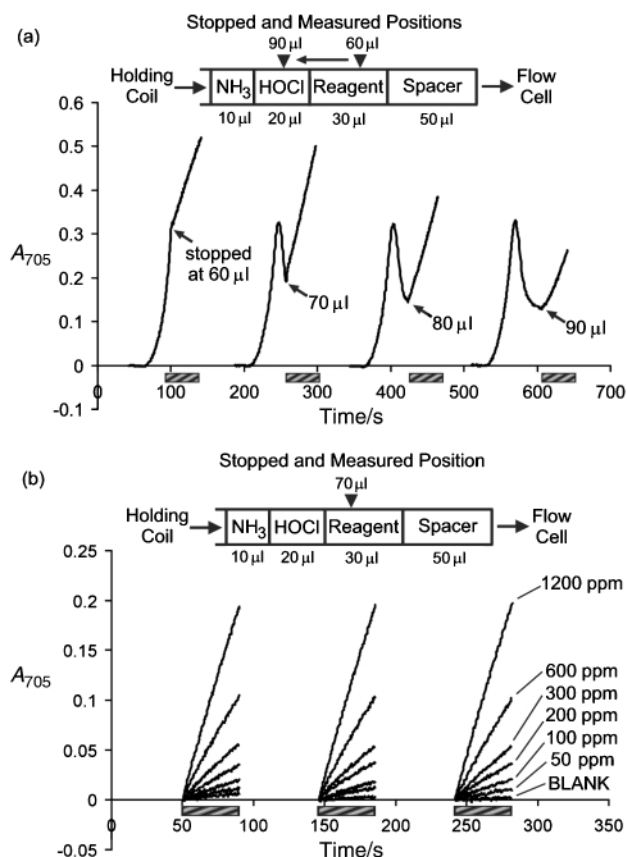
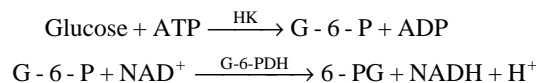


Fig. 2 (a) The stacked zones of sample, reagents and spacer are depicted for the ammonia assay and the slope of kinetic response strength is shown as a function of volumetric displacement of the stacked zones into the detector cell. (b) High reproducibility is observed for the optimized μSI protocol. Kinetic measurements in the 0–1200 ppm range are shown as triplicate injections for each concentration. Hatched rectangles indicate stopped-flow measuring periods.

with $r^2 = 0.9984$ for the concentration range of 20–120 ppm (Fig. 3b).

Glucose protocol



The glucose assay¹⁰ is a two-step reaction, however only a single reagent as a mixture is needed to perform the μSI assay. In Fig. 4, the described stacking sequence used generated a linear response ($r^2 = 0.9994$) for the concentration range of 35–1000 ppm. In this glucose measurement the final product was NADH, which need to be measured at 340 nm using a deuterium lamp source.

Free iron protocol



Using the same sequencing profile as was used in the glucose assay, the sample solution, reagent, and carrier spacer solutions were stacked into the holding coil. However, since the reaction is very fast, no increase in the absorbance signal was observed during a stopped flow period indicating that the reaction was complete (Fig. 5a). Therefore, the free iron measurements were

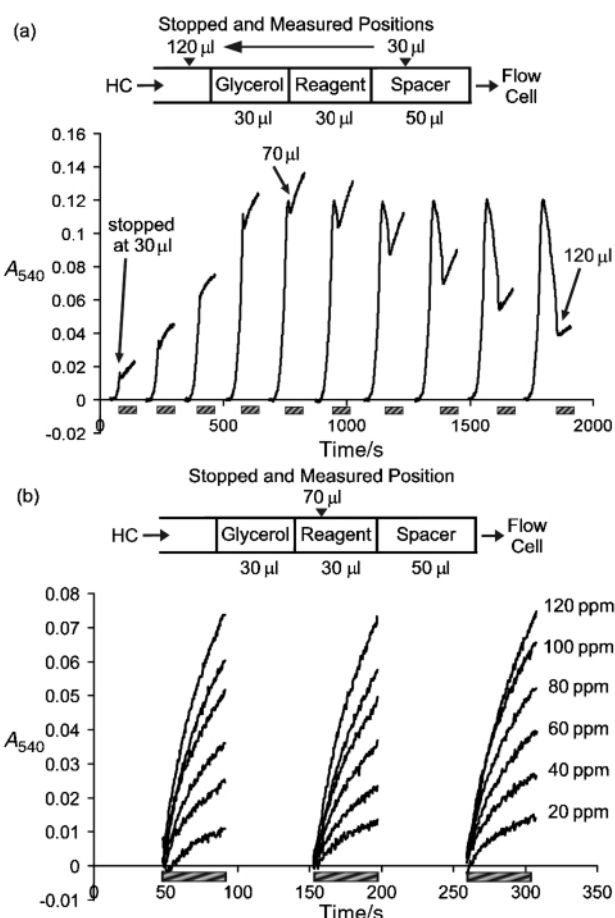


Fig. 3 (a) The stacked zones of sample, reagent and spacer are depicted for the glycerol assay and the slope of kinetic response is shown as a function of volumetric displacement of the stacked zones into the detector cell. (b) High reproducibility is observed for the optimized μSI protocol. Kinetic measurements in the 20–120 ppm range are shown as triplicate injections for each concentration. Hatched rectangles indicate stopped-flow measuring periods.

performed in a continuous flow mode producing a typical transient peak like those observed with traditional FIA assays. A calibration curve (Fig. 5b) using the μ SI instrument in a continuous flow mode at $5 \mu\text{L s}^{-1}$ produced a linear response ($r^2 = 0.9990$) in the 50–400 ppm range. The final reaction product is a ferrozine–Fe complex which is monitored at 560 nm.¹¹

Off-line fermentation

The monitoring of the *E. coli* fermentation was carried out as discrete samples were collected over a 72 h period during which the level of nutrients varied according to fermentation protocol (Fig. 6a). The samples collected were immediately centrifuged and the supernatant to be analyzed later was stored at 5 °C. The lab-on-valve system was calibrated immediately prior and after each analysis run for each individual analyte.

On-line fermentation monitoring

The on-line monitoring of an *E. Coli* culture was carried out over a 4 h period while the fermentation broth was withdrawn continuously from the tank at a flow rate of $\approx 0.5 \text{ mL min}^{-1}$ using a peristaltic pump. From this stream, assay samples were periodically withdrawn for the μ SI instrument and analyzed every 6 min (Fig. 6b). Since ammonia is a critical nutrient, where high or low concentrations may adversely affect the fermentation process, it was selected as the analysis candidate

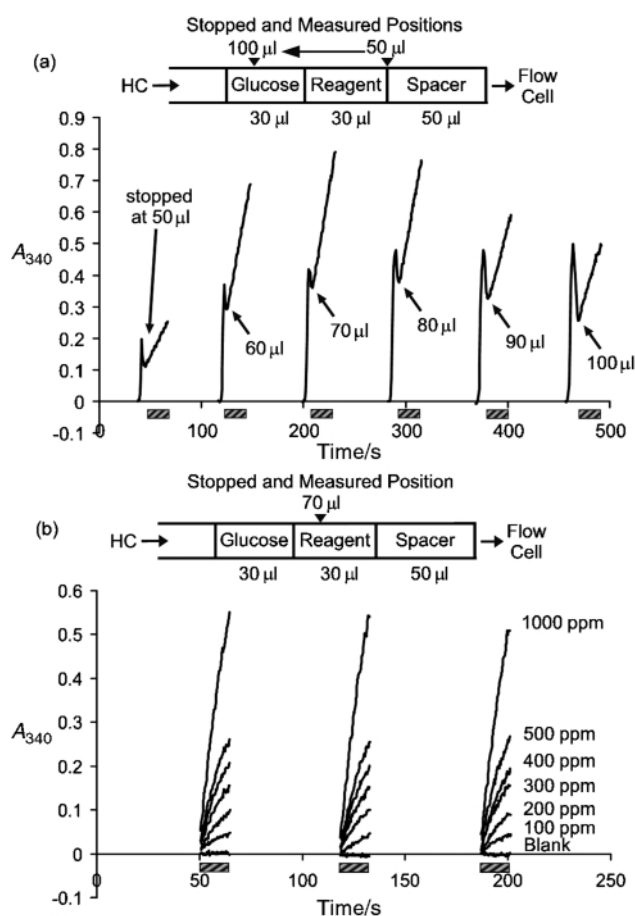


Fig. 4 (a) The stacked zones of sample, reagent and spacer are depicted for the glucose assay and the slope of kinetic response is shown as a function of volumetric displacement of the stacked zones into the detector cell. (b) High reproducibility is observed for the optimized μ SI protocol. Kinetic measurements in the 0–1000 ppm range are shown as triplicate injections for each concentration. Hatched rectangles indicate stopped-flow measuring periods.

for the on-line monitoring. In order to document that the lab-on-valve instrument functioned properly, ammonia standards were periodically injected to validate reliability of the continuous monitoring. The variation in the standards responses over the 4 h period was found to be well within the required precision for on-line fermentation monitoring.

Conclusion

A present weakness in the current lab-on-valve design is that it does not provide thermostat control of the manifold, which is a desirable feature for kinetic based measurements. Fortunately, the ambient temperature of the fermentation laboratory did not vary more than $\pm 1 \text{ }^\circ\text{C}$ during the experiment run which in combination with frequent standard injections assured a satisfactory performance with respect to the accuracy of the measurements. Importantly, the compact μ SI instrument was robust and easily interfaced to a computer and fermentor running reliably without any manual interventions over the course of the day. Over 40 assays were performed during the on-line run generating less than 30 mL of total waste at the end of the fermentation. Also, reagent consumption was substantially reduced. Thus, the amount of ammonia reagent intentional for one manual assay was sufficient for 1000 assays in the μ SI mechanism. Similarly, a glucose kit designed for 67 assays was sufficient for 667 assays in lab-on-valve configuration. Since the instrument including reagent reservoirs is quite compact, the entire unit was easily placed directly adjacent to the fermentor minimizing the distance traveled by the sample from the sampling probe into μ SI system.

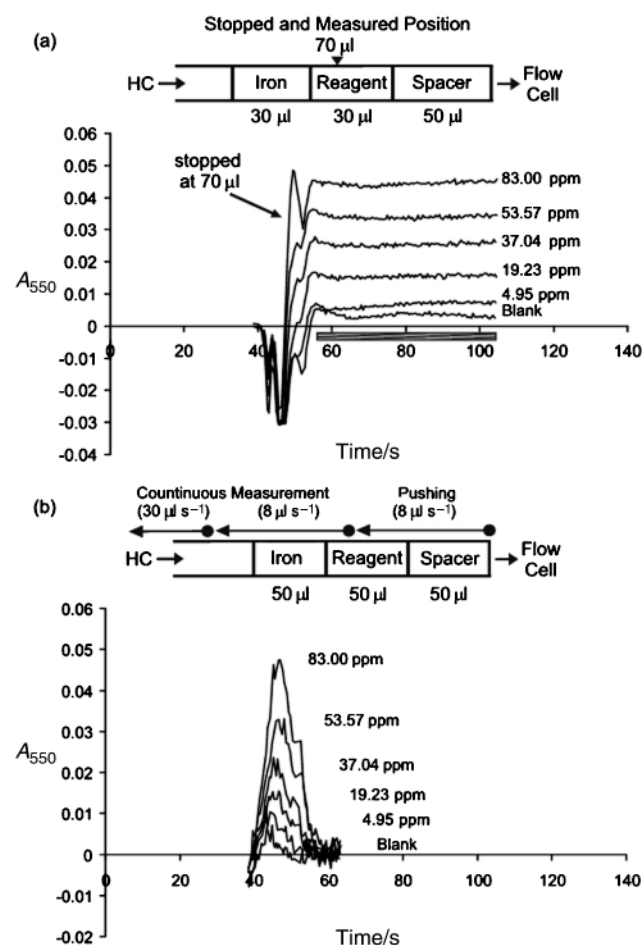


Fig. 5 The stacked zones of sample, reagent and spacer are depicted for the iron assay. (a) Peaks obtained by stopped flow measurements; and (b) continuous flow measurements in the 0–83 ppm concentration range. Hatched rectangles indicate stopped-flow measuring periods.

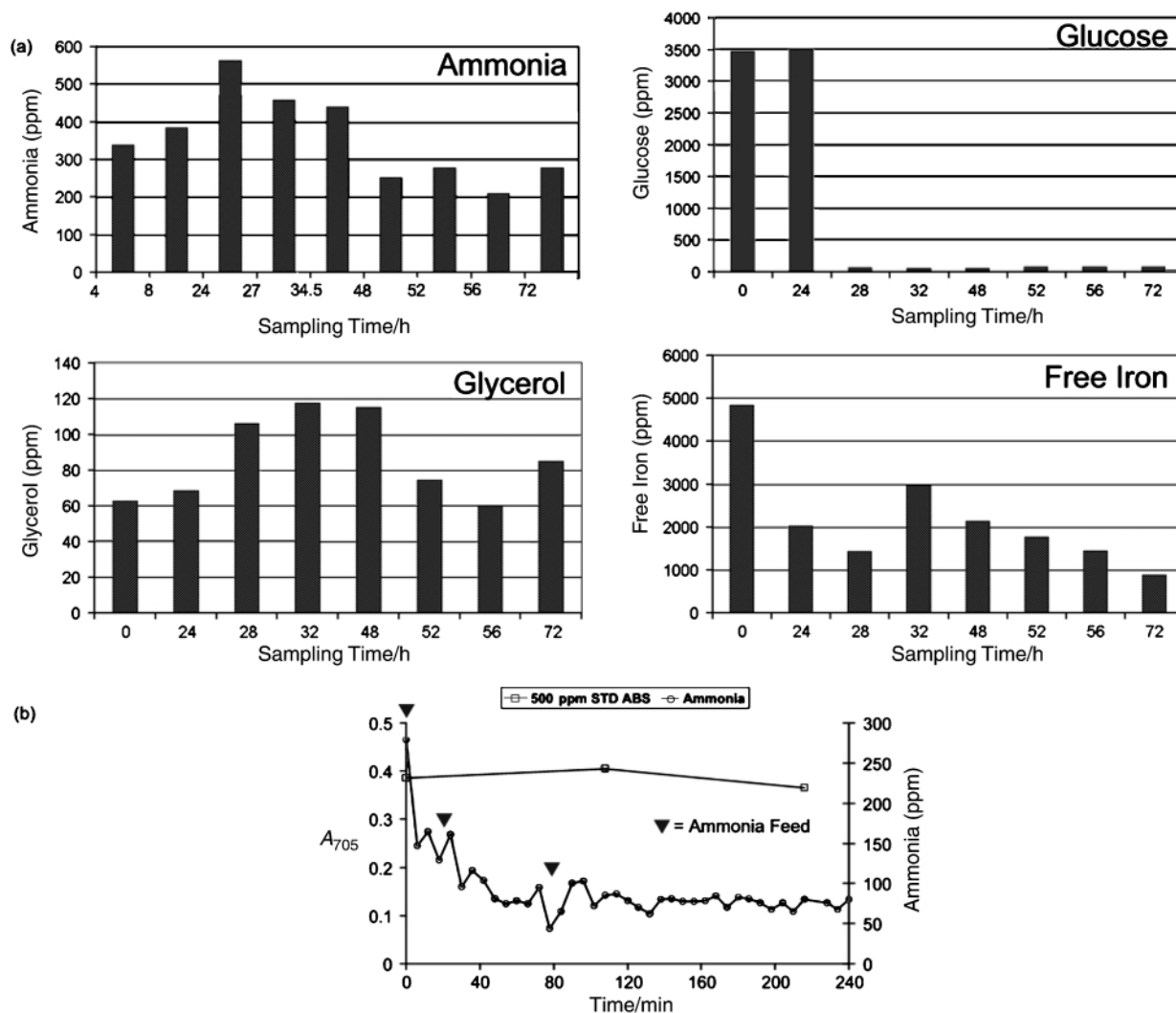


Fig. 6 (a) Off-line measurements of ammonia, glucose, glycerol and free iron show a general depletion of nutrients as the fermentation proceeds in time. (b) On-line monitoring of ammonia for an *E. coli* cell culture is shown. Recalibration of the lab-on-valve system is indicated with the 500 ppm (NH_4) standard.

Thus, the present work is the first practical demonstration of the microfluidic assay carried out within integrated lab-on-valve manifold. These on-line and off-line applications successfully demonstrate the robustness of the μSI instrument, which is constructed from readily available commercial peripherals (pumps, valves, fiber optics and computers), coupled to the mezzofabricated μSI manifold. While present developments in MEMS (μTAS) microchips are always designed to accommodate a specific reagent based assay operating in a classical FI mode, μSI embodied in the lab-on-valve device is designed to accommodate a wide variety of assays since the conditions of assay protocol are varied by changing the software protocol.

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