Published in final edited form as:
Anal Chem. 2011 July 1; 83(13): 5207–5213. doi:10.1021/ac2003938.

Push-pull perfusion sampling with segmented flow for high temporal and spatial resolution in vivo chemical monitoring

Thomas R. Slaney[1], **Jing Nie**[1], **Neil D. Hershey**[1], **Prasanna K. Thwar**[2], **Jennifer Linderman**[2], **Mark A. Burns**[2], and **Robert T. Kennedy**[1,*]

[1] University of Michigan, Department of Chemistry, 930 N. University Ave. Ann Arbor, MI 48109

[2] University of Michigan, Department of Chemical Engineering, 2300 Hayward St., Ann Arbor, MI 48109

Abstract

Low-flow push-pull perfusion is a sampling method that yields better spatial resolution than competitive methods like microdialysis. Because of the low flow rates used (50 nL/min) it is challenging to use this technique at high temporal resolution which requires methods of collecting, manipulating, and analyzing nanoliter samples. High temporal resolution also requires control of Taylor dispersion during sampling. To meet these challenges, push-pull perfusion was coupled with segmented flow to achieve *in vivo* sampling at 7 s temporal resolution at 50 nL/min flow rates. By further miniaturizing the probe inlet, sampling with 200 ms resolution at 30 nL/min (pull only) was demonstrated *in vitro*. Using this method, L-glutamate was monitored in the striatum of anesthetized rats. Up to 500 samples of 6 nL each were collected at 7 s intervals, segmented by an immiscible oil and stored in a capillary tube. The samples were assayed offline for L-glutamate at a rate of 15 samples/min by pumping them into a reagent addition tee fabricated from Teflon where reagents were added for a fluorescent enzyme assay. Fluorescence of the resulting plugs was monitored downstream. Microinjection of 70 mM potassium in physiological buffered saline evoked L-glutamate concentration transients that had an average maxima of 4.5 ± 1.1 μM (n = 6 animals, 3–4 injections each) and rise times of 22 ± 2 s. These results demonstrate that low-flow push-pull perfusion with segmented flow can be used for high temporal resolution chemical monitoring and in complex biological environments.

Introduction

Monitoring neurotransmitters in the brain extracellular compartment is a powerful tool for studying neuronal function and psychological disorders[1–3]. In such measurements, temporal resolution is important because concentration changes of neurotransmitters in the extracellular space around synapses are known to occur in milliseconds to seconds [1, 4]. Spatial resolution is important because the brain contains many small structures with distinct functions, neuronal populations, and neurotransmitter dynamics. Microdialysis sampling coupled to analytical methods is a widely used approach for *in vivo* monitoring. If the sensitivity of the analytical method is sufficient, short sampling intervals can be used and temporal resolution of a few seconds can be achieved[5–8]; however, the relatively large size of the probes, which are typically over 2 mm long and 200 μm diameter, preclude their use for studying smaller brain regions. In this work, we combine the miniaturized sampling method low-flow push-pull perfusion with segmented flow to achieve 7 s temporal

Rtkenn@umich.edu.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

resolution and spatial resolution of 0.016 mm^2 *in vivo*. We further show *in vitro* the potential for sampling at 200 ms resolution with appropriate fluidics.

Push-pull sampling has been used in the brain since 1961[9, 10]. In this method, artificial cerebrospinal fluid (aCSF) is continuously infused into tissue through one tube and withdrawn from a second tube that is placed beside or concentric with the infusion tube. Push-pull sampling fell out of favor because of tissue damage associated with direct tissue contact of fluids flowing at microliter per minute rates. Spatial resolution can be improved and tissue damage greatly reduced by using narrow bore capillaries as the sampler tubing and flow rates less than 50 nL/min[11]. Spatial resolution of low-flow push-pull perfusion is inherently better than microdialysis as the active sampling area is only limited by the spacing of the probe capillary inlets.

Achieving high temporal resolution with low-flow push-pull sampling places great demands on sample manipulation capability, assay throughput, and assay sensitivity. Consider that if sampling at 50 nL/min, then a 60 min experiment at 10 s temporal resolution would require collection and analysis of 360 samples of 8 nL volume each. Furthermore, Taylor dispersion, i.e. zone broadening due to flow and diffusion[12], during sampling can reduce temporal resolution. Initial experiments with low flow push-pull perfusion achieved 5 min temporal resolution by off-line fraction collection and analysis by CE[11]. Coupling low-flow push pull perfusion on-line with CE has allowed 16 s[13] and 45 s[14, 15] temporal resolution sampling from the eye and brain respectively; however, in these cases Taylor dispersion was minimized by using short tubing that would be impractical for freely moving subjects. This approach also precludes off-line analysis which is often advantageous.

In this work, we have coupled low-flow push-pull perfusion to segmented flow to both improve temporal resolution and facilitate manipulation of the nanoliter fractions that are collected. In segmented flow, fractions are collected into a tube or channel as discrete plugs separated by an immiscible carrier phase that wets the tubing surface. As previously shown for microdialysis and “chemistrode” sampling, this approach prevents loss of temporal resolution due to Taylor dispersion during transport to an analytical system[16–18]. This method also provides a convenient way to manipulate nanoliter samples in view of the operations that have been developed for plugs[19–25]. Furthermore, several methods have been developed to analyze plug samples including enzyme assay, immunoassay[26], [2423]electrophoresis[16, 27], and mass spectrometry[28, 29].

A significant challenge for coupling segmented flow with push-pull perfusion is developing flow control for plug formation from the “pull” or sampling capillary. We found that this problem could be solved by using vacuum at the outlet of collection tubing to pull oil and brain perfusate into a tee where flow segmentation occurred. As an initial application of this approach, we demonstrate *in vivo* monitoring of L-glutamate, a primary excitatory neurotransmitter in the brain known to have rapid concentration dynamics[30]. L-glutamate was determined by fluorescent enzyme assay of the plugs in an off-line system. The assay allowed hundreds of samples with ~6 nL volume to be assayed at 15 samples/min. The system is shown to provide an efficient means of collecting, manipulating, and analyzing the large number of low volume samples required for high temporal and spatial resolution sampling.

Materials and Methods

Chemicals and Materials

Unless otherwise specified, all reagents were purchased from Fisher Scientific (Fairlawn, NJ) and were certified ACS grade or better. L-glutamic acid and 1*H*,1*H*,2*H*,2*H*-perfluoro-1-

octanol (PFO) were purchased from Sigma-Aldrich (St. Louis, MO). Perfluorodecalin and FC-72 were purchased from Acros (Morris Plains, NJ). L-glutamate oxidase, glutamate pyruvate transaminase, horseradish peroxidase, L-alanine, dimethylsulfoxide, and Amplex Red were purchased as a kit from Invitrogen (Carlsbad, CA). Fused silica capillaries were purchased from Polymicro (Phoenix, AZ). Teflon PFA tubing was purchased from Upchurch (Oak Harbor, WA). 1/32″ polyvinylidine difluoride (PVDF) sheets were purchased from Small Parts (Lexington, KY).

Probe Fabrication

"Side-by-side" push-pull probes were constructed as described previously[14]. Briefly, two 10 cm lengths of 20 μm inner diameter (ID), 90 μm outer diameter (OD) capillary were inserted through a 27-gauge stainless steel hypodermic needle as illustrated in Figure 1A (BD, Franklin Lakes, NJ). The capillaries were cemented in place by applying cyanoacrylate adhesive to the opposite end of the needle (Duro Super Glue, Henkel, Rocky Hill, CT). To adapt these capillaries to 360 μm fittings, 2 cm lengths of 150 μm ID, 360 μm OD capillary were glued concentrically over the ends of these using thixotropic optical epoxy (353ND-T, Epoxy Technology, Billerica, MA).

For sub-second resolution experiments, plug-generating tees were fabricated from PVDF by using capillaries as molds and a 360 μm drill bit (a detailed procedure is presented in section I of the Supporting Information). The ends of the probe inlet capillary and Teflon storage tubing were placed in close proximity within this tee and oil entered by flowing over the outside of the inlet capillary, as shown in Figure 2A. These probes used a 1 cm long × 10 μm ID aqueous inlet, a 10 cm long × 25 μm ID oil inlet, and a 25 cm long × 50 μm ID Teflon collection tubing. The oil used was 10:1 (v:v) FC-72:PFO, and 290 mm Hg vacuum was applied to operate the probe. Resorufin standards were prepared in 100 mM sodium pyrophosphate, pH 8.6.

Surgical Procedures

All surgical procedures were performed according to a protocol approved by the University Committee on Use and Care of Animals. Male Sprague-Dawley rats between 250 and 350 g were anesthetized by intraperitoneal administration of 75 mg/kg ketamine and 0.25 mg/kg dexmedetomidine. Boosters of 25 mg/kg ketamine and 0.08 mg/kg dexmedetomidine were given as needed. Anesthetized rats were mounted in a stereotaxic frame and the push-pull sampling probe was inserted into the striatum at 1.0 mm anterior and ± 2.6 mm lateral to bregma, and 5.0 mm ventral to the dura. During insertion, both capillaries were flushed with aCSF to prevent clogging as described elsewhere[11]. The aCSF contained 145 mM NaCl, 2.68 mM KCl, 1.01 mM $MgSO_4$, 1.22 mM $CaCl_2$, 1.55 mM Na_2HPO_4, and 0.45 mM NaH_2PO_4, pH 7.4[15]. For K^+ stimulation experiments, a capillary microinjector[31] mounted on a second stereotaxic arm, angled 10° to vertical in the coronal plane, was inserted 0.1 mm right of the sampling probe inlet. The sampling probe was positioned so that the beveled side faced the injector. After probes were inserted, 45 min equilibration time was allowed as preliminary experiments showed that for 30 min after insertion L-glutamate concentrations were not stable. Stimulated L-glutamate release was achieved by infusing 100 nL of high-K^+ aCSF over 6 s. High-K^+ aCSF was prepared the same as aCSF, except with 70.0 mM KCl and 77.7 mM NaCl.

Probe Operation

An overview of the fluidic system for *in vivo* sampling and analysis is shown in Figure 1. To provide the "push" flow, one capillary of the probe was connected to a 25 μL syringe (Gastight, Hamilton Co., Reno, NV) using 360 μm capillary fittings and approximately 30 cm of 40 μm ID capillary, and this syringe was placed in a syringe pump (Fusion 400,

Chemyx, Stafford, TX). After probe insertion, the "pull" capillary was connected to a 100 μm ID tee (C360QTPKG4, Valco Instruments, Houston, TX). One inlet of this tee was attached to a 20 cm length of 40 μm ID capillary which was placed in a vial of a 50:1 (v:v) solution of perfluorodecalin:PFO, the immiscible oil phase. A 50 cm length of 150 μm ID, 360 μm OD Teflon PFA tubing was attached to the third inlet of the tee. 610 mm Hg of vacuum was applied to the outlet of the Teflon tube to provide 50 nL/min of "pull" through the probe inlet and approximately 70 nL/min of oil flow simultaneously. To provide a constant flow rate, this Teflon storage tubing was primed with plugs of aCSF before sampling. To monitor the aqueous flow, plug frequency and quality were visually observed.

After sampling from the brain, the probe was removed and sequentially placed into a series of standards of L-glutamate in aCSF for calibration. To prevent clogging during removal, vacuum was stopped and the "push" flow was increased to 500 nL/min. Standards were sampled without "push" flow to calibrate for absolute recovered concentrations.

Enzyme Assay and Plug Analysis

Plugs were collected into and stored in 150 μm ID by 360 μm OD Teflon tubing. Samples were analyzed 1–3 h after collection, and were stored for this time at ~4°C in a Petri dish. For analysis, the sample plugs were pumped into a Teflon reagent addition tee at 200 nL/min (see Figure 3A) using a syringe pump (Fusion 400, Chemyx, Stafford, TX). (Fabrication of the tee is described in section II and figure S-1 of the Supporting Information.) Enzyme assay reagents were pumped through the orthogonal inlet of the tee at 50 nL/min using another syringe pump yielding a total flow rate of 250 nL/min. The reagent inlet was a 50 μm ID fused silica tube. The hydrophilic material was important to prevent carry-over of sample[23]. PDMS unions consisting of a ~5 mm long × 360 μm ID channel were used to connect Teflon tubings.

L-glutamate assay reagents were prepared to achieve the reaction concentrations recommended by the manufacturer when added at a 1:2 reagent:sample volume ratio. These reagents were prepared as two solutions in 100 mM Tris-HCl pH 7.5, the first containing 0.3 mM Amplex Red, and the second 7.5 U/mL of horseradish peroxidase, 0.24 U/mL L-glutamate oxidase, 15 U/mL glutamate-pyruvate transaminase, and 6 mM l-alanine. These reagents were mixed at a tee fitting while being injected into plugs. The resulting plugs passed out of the third arm of the tee and into a 150 μm ID by 60 cm long Teflon tube. Fluorescence of plugs was measured in this tube 50 cm downstream of the tee as they passed through the detector. The laser-induced fluorescence detector utilized a 543 nm 1.5 mW He-Ne laser (Melles Griot, Carlsbad, CA) for excitation, a 580 nm emission filter (XF3022, Omega Optical, Brattleboro, VT), and a photomultiplier tube for detection.

Safety Considerations

Perfluorinated surfactants have been shown to cause chronic health effects. Proper care and personal protective equipment should be utilized to avoid contact with these liquids.

Results and Discussion

Probe Design

The fluidics for *in vivo* sampling by push-pull perfusion and collecting fractions as plugs are illustrated in Figure 1A. In this system, a syringe pump provides push flow and vacuum pulls both sample from the probe and oil from a reservoir through a tee and into a collection tube. The oil and sampling capillary geometries must be matched to provide appropriate flow and plug generation rates. They also must be selected with practical considerations in mind such as ease of fabrication, pressure drops required, and potential for leaks. Smaller

bore tubes provide less dispersion and better temporal resolution, but they also are more difficult to incorporate into assay systems and require higher pressure drops that are more prone leakage. Thus, these parameters ultimately affect the temporal resolution and robustness of the measurements. (A quantitative description of the trade-offs for different capillary dimensions are discussed in section III and illustrated in figure S-2 of the Supporting Information.)

Based upon these considerations, capillaries with 20 μm ID by 10 cm length and 40 μm ID by 20 cm length were selected for the aqueous phase (sampling) capillary and oil inlet capillary respectively. The collection tubing was 150 μm ID Teflon for *in vivo* studies. This ID tubing is compatible with previously published methods of reagent addition which is necessary for assays[23, 25]. Using this system, regularly shaped plugs were generated as illustrated in Figures 1B and 1C. By measuring the width and frequency of plugs generated, we determined the flow rate of through the sampling capillary to be 48 ± 8 nL/min (n = 5 different probe and tee systems), which is within the desirable flow rates for low-flow push-pull perfusion[11], with 610 mm Hg absolute pressure applied. This vacuum was small enough that failure due to air leaks was eliminated. The flow rate in the collection tubing was 46 ± 5 % aqueous (n = 5) so that the oil spacers and sample plugs were similar widths. Sample plugs were measured to be 5.9 ± 1.2 nL using different probes (n = 5) corresponding to 7 s plugs of 330 μm length.

Spatial resolution is inherently better than microdialysis as the active sampling area is based on the spacing of the two capillary lumen between which the flow occurs. If the active sampling area is assumed equal to the tip surface area of these two capillaries, the sampling region is then approximately 90 μm by 180 μm, an area of about 0.016 mm^2. This area is approximately 80-fold less than the surface (i.e., sampling) area of a relatively small 2 mm long by 200 μm diameter microdialysis probe.

Temporal Resolution Characterization

Temporal resolution in a plug-based system can be defined as $t_{res} \approx \frac{\varphi}{f}$ where φ is the number of plugs required to observe a change (from 10 to 90% of a concentration step), and *f* is the plug frequency (Hz)[17]. As shown in Figure 4A, an L-glutamate step change could be observed with just 1 plug within 150 μm ID Teflon collection tubing for 7 s resolution. Reducing the collection tubing ID to match that of the tee (100 μm) allows smaller, more frequent plugs, occurring at 1.7 ± 0.3 s (n = 3). Step changes of resorufin measured directly by laser induced fluorescence could be resolved within two plugs for 3.4 s resolution. Finite element analysis (not shown) suggested that this resolution was limited by the dead volume of the tee.

To test the limits of temporal resolution of sampling with segmented flow, a PVDF tee with lowered internal volume was fabricated. As shown in figure 2A, this tee allowed the probe capillary outlet to be placed immediately adjacent to the plug storage tubing. A 10 μm ID, 1 cm long sampling capillary was used. A 50 μm bore Teflon storage tubing allowed faster plugs to be stably generated (0.2 s intervals, 0.1 nL volume, n = 3) for a 30 nL/min flow rate. By switching between vials of resorufin, step changes were observed within one plug for a 0.2 s rise time (as shown in Figure 2B). These results show that sub-second temporal resolution is possible by this method of sampling. (These studies did not use a push flow; however, finite element modeling of the system using COMSOL suggested that internal volumes in the pull arm and segmenting tee are the limiting factor in temporal resolution.) For *in vivo* analysis, reagent addition tees for the lower volume plugs and coupling with a push-pull flow would be required.

High Throughput Determination of L-Glutamate in Nanoliter Sample Plugs

The *in vivo* sampling probe used generates 500 plugs of 5.9 nL volume in 1 h. Therefore, the analytical procedure used must have sufficient throughput and sensitivity to analyze the fractions. For this work, we chose a fluorescence enzyme assay for L-glutamate. The reaction scheme of the assay used is:

$$\mathrm{L-glutamate+O_2} \xrightarrow{\text{L-Glutamate Oxidase}} \alpha-\mathrm{ketoglutarate+H_2O_2}$$

$$\mathrm{H_2O_2+Amplex\ Red} \xrightarrow{\text{Horseradish Peroxidase}} \mathrm{O_2+resorufin}$$

$$\alpha-\mathrm{ketoglutarate+L-alanine} \xrightarrow{\text{Glutamate-Pyruvate Transaminase}} \mathrm{L-glutamate+pyruvate}$$

where resorufin is the detected fluorescent product. This assay is attractive as it incorporates an enzyme (glutamate-pyruvate transaminase) to regenerate L-glutamate and amplify signal. Endogenous hydrogen peroxide may contribute some background to this assay, as it has been observed in the striatum[32]. Reaction time (35 min) was chosen based on manufacturer-recommended times and was verified experimentally to provide adequate sensitivity to observe sub-micromolar concentration changes (see Figure 4B).

To perform the assay on plugs, the fluidic system illustrated in Figure 3 was used. Reagents were reproducibly added to plugs with little cross-contamination by pumping them through a hydrophilic capillary that intersects the plug flow path in a hydrophobic tee similar to that described before[23, 25]. Previous work for reagent addition to pre-formed plugs has used tees fabricated in PDMS; however, because perfluorinated oils have low energy of interaction with PDMS, the surface had to be modified to prevent aqueous plugs from coalescing and splitting[33]. We found that although such modifications were suitable for *in vitro* studies, they were not sufficiently stable when exposed to samples collected *in vivo*. Therefore, we developed a reagent-addition tee using 150 μm ID Teflon tubing as shown in Figure 3. Surface stability of this Teflon tee was sufficient to allow it to be reused for many experiments.

Using this tee, approximately 3 nL of reagent was added to each of the plug samples as they passed the tee junction. The resulting fluorescence was detected ~50 cm downstream, corresponding to a 35 min reaction time. At the flow rates used, samples were assayed at ~4 s intervals (15 samples/min) after the initial 35 min of incubation. Up to 500 sample and standard plugs were analyzed in one 75 min session demonstrating the stability of this high throughput system. A fluorescence trace during calibration of the system is shown in Figure 4A. For calibration, L-glutamate was spiked into aCSF in steps from 0 to 10 μM while sampling without “push” flow. The fluorescence changes are linear with concentration as shown in Figure 4B. (A non-zero intercept was typically observed with calibration because of background oxidation of Amplex red to resorufin. This effect can be minimized by cooling the reagent supply and mixing it with horseradish peroxidase just at the time of reagent addition.) The step changes were detected across 1–2 plugs, indicating that temporal resolution was preserved despite the long incubation time. Separate experiments showed that the presence of “push” flow did not affect the temporal resolution.

The system provided a convenient and automated way to manipulate and analyze the 5.9 nL plugs collected from the probe while maintaining temporal resolution and providing good throughput. Alternatives to the segmented flow approach would be to collect individual fractions into a multi-well plate or similar device; however collecting 5.9 nL fractions in a multi-well plate at high temporal resolution, storing them, and adding appropriate reagent amounts would be challenging. Another alternative would be to operate a continuous flow assay; however, this would greatly compromise temporal resolution. A direct comparison of

continuous flow and segmented flow during calibration is shown in Figure 4A. Due to dispersion caused by flow and diffusion during transfer within the storage tubing (100 μm ID), 5 min concentration steps produced a continuous increase in detected signal instead of the sharp steps observed with segmented flow.

In Vivo Monitoring of L-glutamate

We used this method to measure L-glutamate in the striatum of anesthetized rats as a demonstration of the potential for *in vivo* monitoring. Average basal L-glutamate concentration was 0.9 ± 0.2 μM (n = 8). For comparison, we measured 1 μL perfusate fractions using a capillary electrophoresis (CE) system described previously[34] and found basal glutamate to be 1.4 ± 0.1 μM (n = 3). We attribute this difference to components of the dialysate that affect assay sensitivity, which decreased the enzyme assay sensitivity at *in vivo* concentrations (not shown). The *in vitro* recovery of our probe was measured to be 57 ± 15 % (n = 3) by sampling resorufin with and without "push" flow (2 μM in aCSF at 37°C). Correcting the CE-measured basal concentration for this recovery gives 2.5 ± 0.2 μM L-glutamate, which agrees with previously reported values of 2.0 ± 0.7, 1.9 ± 0.6, and 2.3 ± 1.2 μM for low-flow push-pull perfusion measurements of L-glutamate[11, 14, 15].

To demonstrate monitoring of rapid concentration changes, we microinjected 100 nL of 70 mM K^+ to evoke brief pulses of L-glutamate efflux. As shown by the example traces in Figures 5A and 5B, such injections resulted in transient increases in L-glutamate concentration recorded by measuring the fluorescence of plugs with reagent added. Average concentration maxima for the first microinjection was 5.6 ± 2.0 μM (n = 6 rats) and 4.0 ± 0.9 μM for subsequent injections in the same animal (n = 6 rats, 2–3 injections each) spaced 5 or 10 min apart. The overall average was 4.5 ± 1.1 μM (n = 6 rats, 21 total injections). For a measure of evoked L-glutamate collected by the probe, area under the curve was calculated for each microinjection. Average peak area for the first stimulation was 121 ± 41 μM·s (n = 6), and 92 ± 16 μM·s for subsequent peaks as shown in Figure 5C (n = 6 rats, 2–3 injections each), an overall average of 102 ± 23 μM·s (n = 6 rats, 21 total injections). The average release of L-glutamate from the first microinjection was not significantly higher than for subsequent by either metric (t-test, $p > 0.05$).

To illustrate that the responses were not an artifact of the microinjection, we repeated the microinjections with normal aCSF and observed no response as shown in Figure 5D (solid line). If glutamate oxidase was omitted from the enzyme mixture, no response to potassium microinjections was observed, indicating transients are changes of glutamate and not hydrogen peroxide (Figure 5D, dashed line). These results are comparable to the 5.7 μM maxima observed in previous work with L-glutamate microelectrodes where 100 nL of 70 mM high-K^+ aCSF was microinjected into the striatum[30].

As shown in Figure 5A, the rise to maximal response for high-K+ aCSF stimulation could be observed within 1 to 2 plugs. Achieving a 1 plug rise for all stimulations would require timing microinjections to be in phase with plug formation. In these preliminary experiments, the average number of plugs required was 2.8 ± 0.3 corresponding to a rise time of 22 ± 2 s (n = 21 injections in 6 rats). Some *in vivo* experiments experienced temporal resolution loss due to plugs coalescing during analysis as shown in Figure 5B (2–3 plugs required for rise time). A syringe driver was used for microinjections, at the disadvantage of requiring at least 6 s to infuse the full volume. A gas pressure microinjection system has allowed 1.6 s rise times to be observed in a comparable microelectrode study[30].

This temporal resolution is comparable to the "state-of-the-art" in microdialysis temporal resolution, while sampling from at least an 80-fold smaller area of the brain. The *in vitro* experiments shows temporal resolution that approaches what is possible with "chemistrode"

sampling[17]. In the chemistrode, plugs are preformed and then passed through the sampling region. The present work shows that with relatively low dispersion in the sampling channel, 200 ms resolution is possible which is comparable to 50 ms achieved for the “chemistrode”. The push-pull system achieves this resolution at much lower flow rates relative to the “chemistrode”, which may be advantageous in sampling delicate tissues that might be disrupted by shear forces, and without concern of contaminating the sample with oil.

Segmented-flow coupled to push-pull sampling may also be compared to biosensors for *in vivo* monitoring. The segmented-flow method provides temporal and spatial resolution that approaches that of the best sensors. Glutamate enzyme sensors with 50 μm by 150 μm dimensions[35], compared to 90 × 180 μm capillary tips used here for sampling, and 0.8 s temporal resolution compared to 7 s here have been reported[30]. Aside from these issues, sensors presently provide advantages of ease of use (e.g., commercial products) and real time monitoring. In contrast, the sampling method should provide better opportunities for monitoring different compounds and multiple compounds simultaneously. In short, this approach to sampling closes the gap in spatial and temporal resolution performance between sampling and sensor methods, but each approach to *in vivo* monitoring retains distinct advantages.

Future Directions and Potential

A number of improvements and other applications seem feasible based on these initial results. This work used an enzyme assay to monitor L-glutamate in 7 s plugs. Development of a smaller reagent addition tee will allow assays to be performed on 200 ms plugs collected from push-pull sampling for detecting more rapid concentration changes. Other analytes could be targeted by using different enzyme systems. The resulting samples are also compatible with a variety of other plug manipulation and analysis techniques such as the “SlipChip” for handling arrays of nanoliter samples[36], immunoassays[26], capillary electrophoresis[16, 27, 37], electrochemical detection[38, 39], and mass spectrometry[28, 29]. While *in vivo* neurotransmitter sampling was the goal of the work described here, segmented flow-coupled low-flow push-pull or direct sampling is adaptable to other applications such as spatially resolved sampling of secretions from tissues *in vitro* (e.g., brain slices or cultured cells).

In the course of these experiments, we observed several difficulties that will need to be addressed for future development. During the sampling procedure, 23% of probes were found to clog, a concession for the benefit of improved recovery granted by having the probe inlet directly in contact with the tissue. Figure 5B shows an experiment where clogging occurred briefly during the third microinjection resulting in a delayed appearance of the transient. Future work will investigate whether different probe designs are less susceptible to clogging. Another practical issue to address is materials and connections. Teflon tubing as the basis of assay fluidics proved to be robust as reagent addition tees could be used for many experiments. However, care in making connections between Teflon tubings was necessary to avoid trapping dust and debris, which could cause plug coalescence and compromise temporal resolution. Additionally, PDMS unions used exhibited a poor tolerance for high backpressure. Future work will focus on development of more reliable unions for analysis. Despite these obstacles, 74% of the sample tubes collected were successfully analyzed (n = 34 experiments). While this enzyme assay provided facile plug analysis and allowed visualization of glutamate transients, its non-specificity may be problematic if quantitative measurements are desired due to matrix interferences including ascorbic acid and hydrogen peroxide.

Conclusions

A method for high temporal resolution sampling using segmented flow coupled to a push-pull sampling probe was demonstrated with an 80-fold spatial resolution improvement over microdialysis. The method provides spatiotemporal resolution for neurotransmitter measurement that approaches what has been achieved with electrochemical sensors, but as a sampling method it is adaptable to other segmented flow-based analyses to provide good versatility in chemical monitoring. The simplicity of the probes, the small dimensions of inlets, and high temporal resolution while maintaining picoliter to nanoliter volumes provides new opportunities for chemical monitoring by sampling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was funded by NIH R37 EB003320 and a strategic alliance grant from Pfizer. We thank Claire Chisolm for assistance with finite element analysis.

References

1. Robinson DL, Hermans A, Seipel AT, Wightman RM. Chem Rev. 2008; 108:2554–2584. [PubMed: 18576692]
2. Watson CJ, Venton BJ, Kennedy RT. Anal Chem. 2006; 78:1391–1399. [PubMed: 16570388]
3. Weiss DJ, Lunte CE, Lunte SM. TrAC, Trends Anal Chem. 2000; 19:606–616.
4. Venton BJ, Robinson TE, Kennedy RT. J Neurochem. 2006; 96:236–246. [PubMed: 16300631]
5. Huynh BH, Fogarty BA, Martin RS, Lunte SM. Anal Chem. 2004; 76:6440–6447. [PubMed: 15516139]
6. Lada MW, Vickroy TW, Kennedy RT. Anal Chem. 1997; 69:4560–4565. [PubMed: 9375517]
7. Parrot S, Sauvinet V, Riban V, Depaulis A, Renaud B, Denoroy L. J Neurosci Methods. 2004; 140:29–38. [PubMed: 15589331]
8. Tucci S, Rada P, Sepúlveda MJ, Hernandez L. J Chromatogr B. 1997; 694:343–349.
9. Gaddum JH. J Physiol (Lond). 1961; 155:1P–2P. [PubMed: 13766987]
10. Myers RD, Adell A, Lankford MF. Neurosci Biobehav Rev. 1998; 22:371–387. [PubMed: 9579326]
11. Kottegoda S, Shaik I, Shippy SA. J Neurosci Methods. 2002; 121:93–101. [PubMed: 12393165]
12. Aris R. Proc R Soc London, Ser A. 1956; 235:67–77.
13. Patterson EE II, Pritchett JS, Shippy SA. Analyst. 2009; 134:401–406. [PubMed: 19173069]
14. Cellar NA, Kennedy RT. Lab Chip. 2006; 6:1205–1212. [PubMed: 16929400]
15. Cellar NA, Burns ST, Meiners JC, Chen H, Kennedy RT. Anal Chem. 2005; 77:7067–7073. [PubMed: 16255611]
16. Wang M, Slaney T, Mabrouk O, Kennedy RT. J Neurosci Methods. 2010; 190:39–48. [PubMed: 20447417]
17. Chen D, Du W, Liu Y, Liu W, Kuznetsov A, Mendez FE, Philipson LH, Ismagilov RF. Proc Natl Acad Sci USA. 2008; 105:16843–16848. [PubMed: 18974218]
18. Wang M, Roman GT, Schultz K, Jennings C, Kennedy RT. Anal Chem. 2008; 80:5607–5615. [PubMed: 18547059]
19. Song H, Tice JD, Ismagilov RF. Angew Chem Int Ed. 2003; 42:768–772.
20. Adamson DN, Mustafi D, Zhang JXJ, Zheng B, Ismagilov RF. Lab Chip. 2006; 6:1178–1186. [PubMed: 16929397]
21. Nie J, Kennedy RT. Anal Chem. 2010; 82:7852–7856. [PubMed: 20738106]
22. Zheng B, Tice JD, Roach LS, Ismagilov RF. Angew Chem Int Ed. 2004; 43:2508–2511.

23. Song H, Li HW, Munson MS, Van Ha TG, Ismagilov RF. Anal Chem. 2006; 78:4839–4849. [PubMed: 16841902]
24. Song H, Chen DL, Ismagilov RF. Angew Chem Int Ed. 2006; 45:7336–7356.
25. Li L, Boedicker JQ, Ismagilov RF. Anal Chem. 2007; 79:2756–2761. [PubMed: 17338503]
26. Liu W, Chen D, Du W, Nichols KP, Ismagilov RF. Anal Chem. 2010; 82:3276–3282. [PubMed: 20334360]
27. Roman GT, Wang M, Shultz KN, Jennings C, Kennedy RT. Anal Chem. 2008; 80:8231–8238. [PubMed: 18831564]
28. Pei J, Li Q, Lee MS, Valaskovic GA, Kennedy RT. Anal Chem. 2009; 81:6558–6561. [PubMed: 19555052]
29. Hatakeyama T, Chen DL, Ismagilov RF. J Am Chem Soc. 2006; 128:2518–2519. [PubMed: 16492019]
30. Day BK, Pomerleau F, Burmeister JJ, Huettl P, Gerhardt GA. J Neurochem. 2006; 96:1626–1635. [PubMed: 16441510]
31. Kulagina NV, Shankar L, Michael AC. Anal Chem. 1999; 71:5093–5100. [PubMed: 10575963]
32. Kulagina NV, Michael AC. Anal Chem. 2003; 75:4875–4881. [PubMed: 14674466]
33. Roach LS, Song H, Ismagilov RF. Anal Chem. 2005; 77:785–796. [PubMed: 15679345]
34. Bowser MT, Kennedy RT. Electrophoresis. 2001; 22:3668–3676. [PubMed: 11699904]
35. Burmeister JJ, Pomerleau F, Palmer M, Day BK, Huettl P, Gerhardt GA. J Neurosci Methods. 2002; 119:163–171. [PubMed: 12323420]
36. Du W, Li L, Nichols KP, Ismagilov RF. Lab Chip. 2009; 9:2286–2292. [PubMed: 19636458]
37. Chiu D. Anal Bioanal Chem. 2010; 397:3179–3183. [PubMed: 20383695]
38. Han Z, Li W, Huang Y, Zheng B. Anal Chem. 2009; 81:5840–5845. [PubMed: 19518139]
39. Liu S, Gu Y, Le Roux RB, Matthews SM, Bratton D, Yunus K, Fisher AC, Huck WTS. Lab Chip. 2008; 8:1937–1942. [PubMed: 18941696]

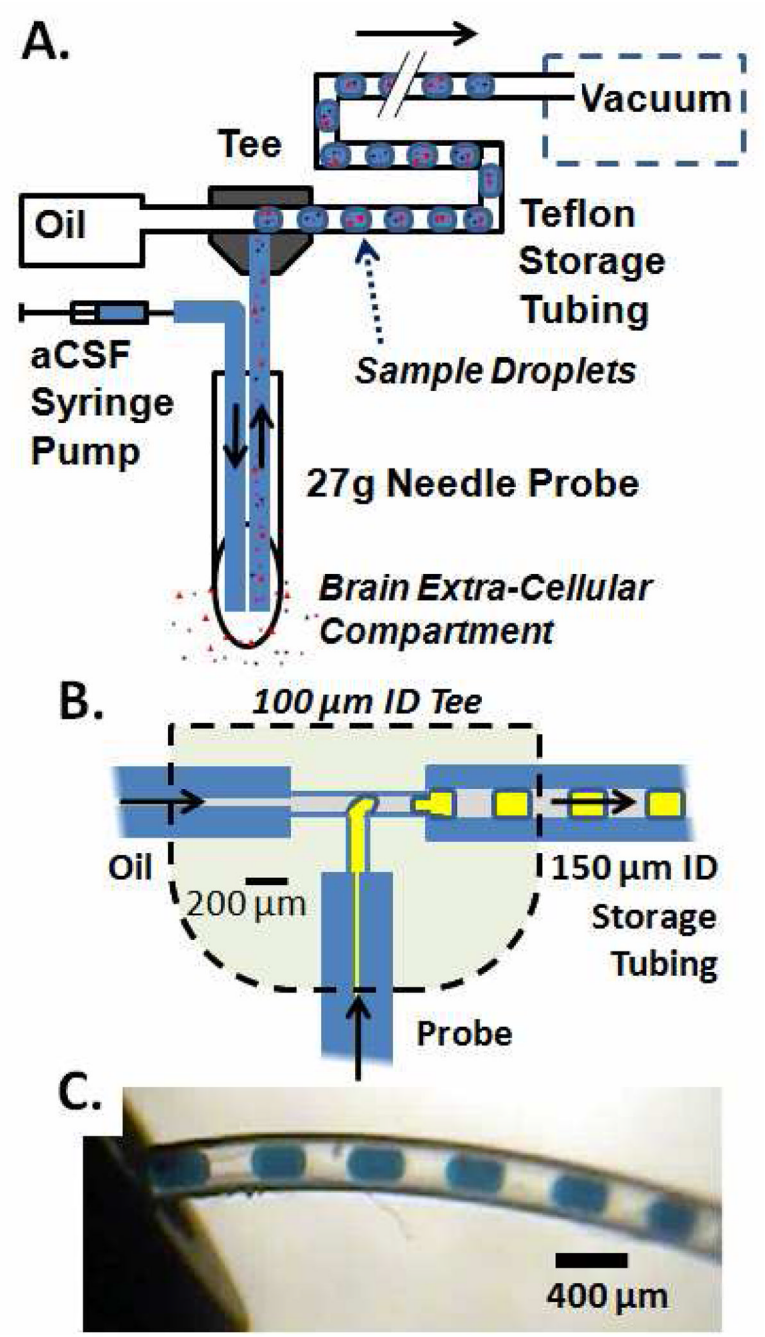

Figure 1.

(A) Diagram of segmented flow-coupled low-flow push-pull probe. Artificial cerebrospinal fluid (aCSF) is infused directly into the tissue, while simultaneously extracellular fluid is withdrawn at an equal flow rate. Suction for sampling flow is generated by vacuum applied to the outlet of Teflon storage tubing, which also pulls the oil to generate the segmented sample stream. (B) Diagram of the internal tee geometry (tee outline not to scale). (C) Photograph of 6 nL sample segments (blue food dye) entering the storage tubing at the tee outlet.

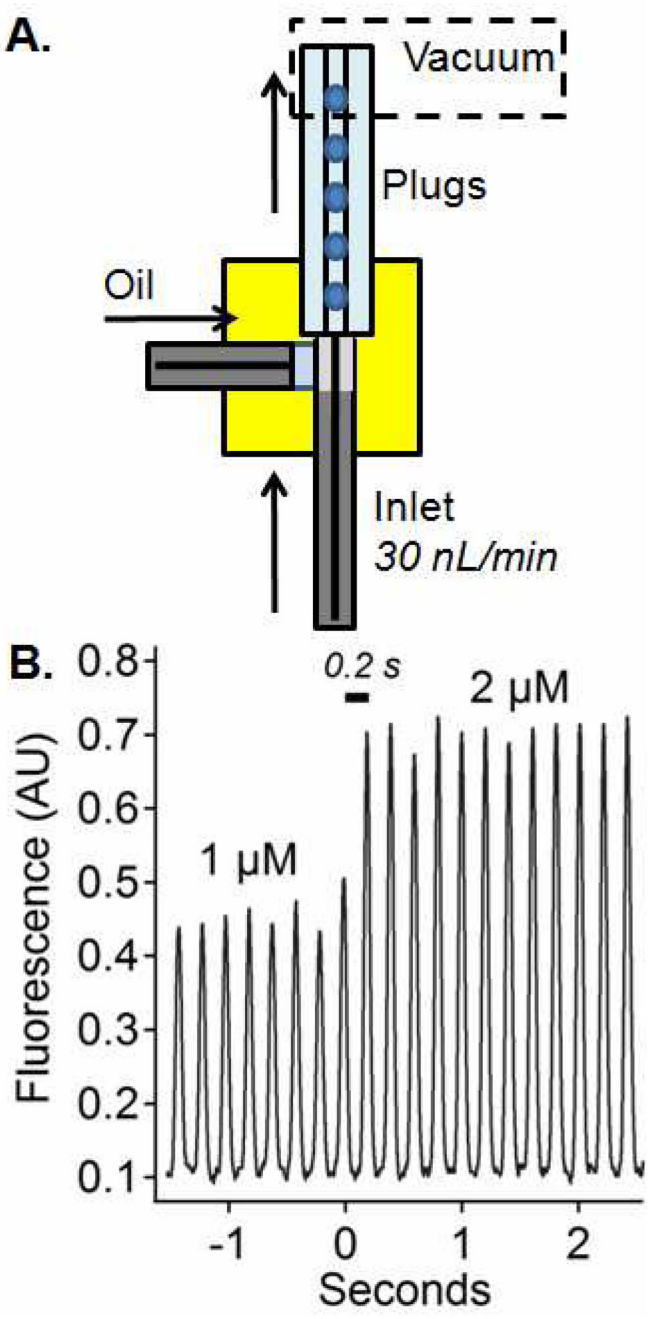

Figure 2.

System for high temporal resolution sampling. (A) A tee from PVDF was molded so to place the probe inlet adjacent to the segmentation tubing. Oil enters the tee by flowing over the end of the inlet capillary within the tee. (B) Fluorescence of plugs in non-fluorescent oil was measured while switching between approximately 1 and 2 µM resorufin standards, demonstrating 0.2 s temporal resolution (1 plug to observe a 10–90% concentration change, 30 nL/min sampling rate).

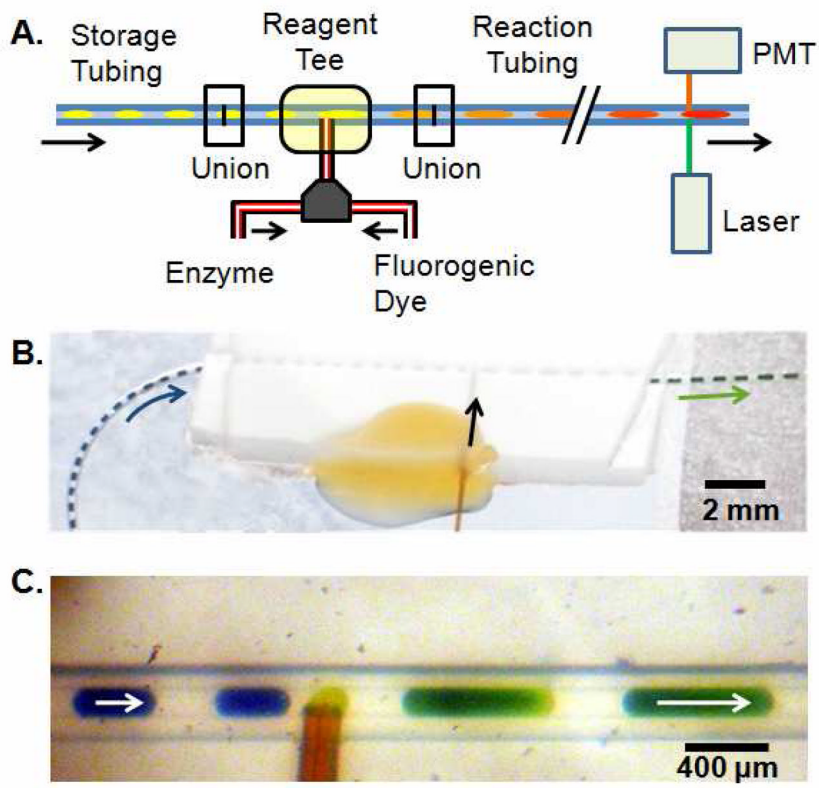

Figure 3.

Operation of a Teflon-based reagent addition tee. (A) Scheme for enzyme assay analysis setup with reagent-addition tee. A 150 µm ID, 360 µm OD Teflon storage tube containing the push-pull sample plugs is connected to the inlet of the reagent-addition tee with a PDMS union. Stored plugs are pushed by a syringe pump through the tee, where the assay reagents (enzyme and fluorogenic dye) are added at an approximately 1:2 reagent:sample volume ratio. These plugs travel through Teflon for approximately 35 minutes while the enzyme reaction incubates. Fluorescence is continuously measured with a green HeNe laser and photomultiplier tube. (B) Photograph illustrating passive tee operation. For visualization, yellow food coloring (from bottom) was added to blue food coloring plugs (left) to yield green plugs (right). (C) Micrograph of reagent addition in the tee as described in B. Arrows indicate flow direction.

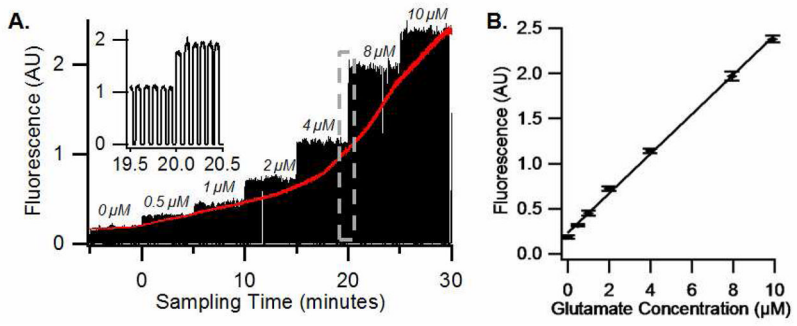

Figure 4.

Calibration curve of recovered L-glutamate concentrations. A stirred vial of aCSF was sampled without push flow, and was spiked at 5 minute intervals from 0 to 10 μM L-glutamate. Resulting plugs were analyzed using the system shown in Figure 3. (A) Recording of fluorescence of plugs for sampled step changes in L-glutamate concentration (black line). A comparable experiment without segmented flow revealed the loss of temporal associated with continuous flow (red line). (B) Calibration curve resulting from sampling experiments. Each point is the average of ~56 standard plugs with 1 standard deviation as the error bar.

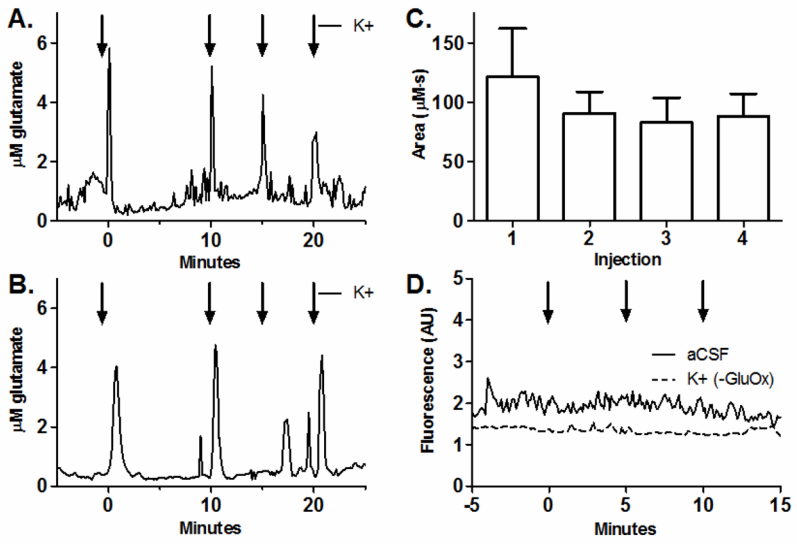

Figure 5.

Segmented flow-coupled low-flow push-pull sampling of neurotransmitters in the rat striatum. A) Measured striatal L-glutamate, with 100 nL 70 mM K^+ aCSF injected at times indicated by the arrows. B) Same as A, but where brief probe occlusion occurred near third injection. C) Mean evoked peak area of repeat injections in 5 rats, (4 rats for injection 4) with SEM. D) Control injections of aCSF (solid), and 70 mM K+ aCSF (dashes) analyzed without glutamate oxidase(arrows indicate approximate times).