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Continuous and Quantitative Delivery of Molecules into Individual Cells with a Diffusional Microburet

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Abstract

Direct delivery of molecules into the cytosol of live cells is required in many areas of biology and clinical research. Molecules of interest include indicator dyes, biomolecules, and pharmacological agents. In this work we describe continuous delivery of molecules into single cells using a Diffusional Microburet, DMB. The DMB is a pulled glass micropipette with a fine tip that contains a microscopic plug made of a hydrogel such as agar or polyacrylamide. This plug prevents flow, but allows diffusive delivery of the molecule of interest from the DMB body into the cytosol, driven by its concentration gradient. This leads to a scheme of sustained intracellular dosing that is highly reproducible and quantifiable, yet does not require the addition of solution volume to the cell. Potential loss of biomolecules from the cytosol through the plug of the DMB can be greatly reduced by proper choice of the pore size and tortuosity of the hydrogel in the DMB tip. The intracellular concentration of fluorescent molecules during delivery can be obtained calibration free. In this work we demonstrate dosing of Lucifer Yellow CH, LY, a charged fluorescent dye, into individual a7r5 vascular smooth muscle cells with a DMB. New types of quantitative analytical experiment on single live cells that the DMB technology enables are titration of intracellular ions and ligands, binding sites, and efflux pathways such as those that are involved in drug resistance.

INTRODUCTION

Direct delivery of molecules into microscopic solution volumes has significance in the chemical analysis of microsamples, in the study of physical chemistry of microscopic domains, and in the manipulation of the solute contents of live cells. Earlier, we tested the use of diffusion through a hydrogel plug placed in the tip of a Diffusional Microburet (DMB) to achieve continuous and quantitative delivery of molecules into aqueous microscopic droplets¹⁻⁸. In this work we describe the adaptation of this approach to biological studies conducted at the single cell level where the methodology enables continuous intracellular dosing of individual cells and connected cell clusters.

The most common method for intracellular delivery of molecules is incubation. Other “bulk” loading methods include scrape loading and electroporation. Incubation is restricted to molecules to which the cell membrane is permeable, or can be permeabilized. Molecules that can be loaded into cells with incubation include the acetoxymethyl ester forms of ion chelators and fluorescent dyes⁹. The delivered molecules may be subsequently retained due to a change in their chemical structure (e.g., charge). Many pharmacological agents and certain drug

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molecules can also be loaded into cells via spontaneous uptake^{10, 11}. A benefit of the incubation method is that the final concentration in individual cells within the population is fairly even. The delivery rate is controlled by passive diffusion across the plasma membrane and in some cases active uptake, and the rate of any subsequent processing as in the case of acetoxymethyl esters. In scrape loading, electroporation, and sonoporation pores are created temporarily in the cell membrane by mechanical, electrical, or acoustic stress, respectively¹²⁻¹⁴. These methods can deliver a wide variety of molecules including macromolecules into the cytosol of cells; the amount delivered into any given cell, however, cannot be controlled. In general, the intracellular concentration of molecules delivered by physical-based methods follows a distribution within the cell population.

Methods for delivery of a bolus of molecules into selected individual target cells include mechanical microinjection and iontophoretic injection. Virtually any molecule can be mechanically injected into the cytosol¹⁵. This requires, however, high pressure to overcome the hydrodynamic resistance of the pipette tip. This necessitates the use of a relatively wide tip that increases the likelihood of cell damage. Injection will also induce mechanical stress due to the added volume, and it is difficult to precisely control the delivered amount. The high pressure required when a narrow tip is used has been shown to be achievable using heat expansion of an alloy in the body of the pipette¹⁶. A particularly innovative approach recently reported by Mirkin *et al.*¹⁷ uses an immiscible liquid|liquid interface to generate high pressure in the tip of the micropipette by varying the potential drop across the interface. The tip of the “attosyringe” can be repeatedly filled with extremely small volumes of reagents by reversing the potential across the interface. Further techniques include carbon nanotubes that can carry molecules attached to their surface inside the cytosol¹⁸. This method causes negligible damage to the plasma membrane, but the type of molecules that can be delivered is limited by the available surface chemistries for reversible immobilization onto the nanotube. Iontophoretic injection¹⁹ is restricted to charged particles and will potentially cause protein redistribution inside the cell.

Delivery by injection methods is discrete. Continuous intracellular delivery of different molecules such as fluorescent tracers is also used in cellular studies. Sustained delivery has been achieved with whole cell patch clamp²⁰ which, however, leads to rapid dialysis of the cell and the loss of vital biomolecules from the cytosol into the micropipette. Also, the patch pipette cannot be removed from the cell during the experiment. The use of an open-tip pipette without active pressure injection is complicated by possible spontaneous convection between the cell and the micropipette that may be caused by the presence of a net hydrostatic pressure, or eventual osmotic and density gradients.

We have shown that adjustable and accurately known delivery of molecules into microscopic aqueous droplets of the size of typical biological cells can be achieved with a Diffusional Microburet, DMB¹⁻⁸. The DMB is an ordinary micropipette but its tip contains a plug made of a hydrogel such as agar or polyacrylamide. This plug prevents convective flow but allows diffusive delivery of molecules from the pipette body. Precise delivery is possible due to the perfect reproducibility of diffusive transport as long as the initial and boundary conditions are the same. The delivery rate can be adjusted by varying the concentration of the molecule that needs to be delivered in the body of the DMB, the pore size of the plug, and/or the diameter of the tip. Diffusion through the hydrogel plug is rate limiting, and the loss of material from the macroscopic DMB body into the microscopic cell is negligible. These two circumstances lead to a quasi-steady-state mode of operation². With this simple tool quantitative analysis of microscopic droplets using diffusional titrations¹⁻³, addition and subtraction of reagents to create and study microreactions^{4, 5}, absorption spectrometry⁶, fluorescence inner filter effects⁷, experiments using fluorescence for assessing processes at aqueous/apolar immiscible interfaces⁸, and electrochemical measurements²¹ have been performed in nano-, pico- and

femtoliter solution domains with accuracy and precision that are on a par with those of ordinary laboratory tools and instruments that operate on scales many orders of magnitude larger.

In this work we tested the applicability of the DMB for dosing molecules of interest into individual target cells; schematics of the technique are shown in Figure 1. Benefits of using a DMB for intracellular delivery include that molecules are added without adding volume to the cytosol. This will prevent dilution or pressure buildup in the cell. The gel plug would also prevent the loss of molecules from the cytosol that are similar in size or larger than the pore size of the hydrogel. The rate of dialysis of smaller molecules will also be significantly diminished as compared to using an open tip patch pipette, due to tortuosity of diffusion paths in the hydrogel. At the same time the rate of influx of the delivered molecule can be made much greater than the rate of loss of similar sized molecules from the cytosol. This can be achieved by adjusting the concentration in the DMB body to a level that is high enough to ensure the desired rate of delivery of the molecule of interest.

The DMB can be used to implement different methodologies of analytical chemistry to probe single live cells in diverse areas of biology where new types of experiment can be designed using continuous and quantitative intracellular delivery of molecules. Such experiments include quantitative titration of intracellular ions and ligands, binding sites, and efflux pathways such as those that are involved in drug resistance.

Here, we use Lucifer Yellow, LY, a fluorescent indicator molecule to demonstrate that quantitative intracellular delivery is feasible with the DMB. LY is a dye often used as a fluorescent tracer molecule in studies of gap junctions between connected cells such as vascular smooth muscle cells and kidney epithelial cells. Due to its charge LY molecules cannot penetrate the cell membrane. Therefore, to deliver LY into a target cell an open tip micropipette with pressure injection^{22, 23} or perfusion with a patch pipette^{24, 25} is conventionally used. We show that the DMB can be used for sustained delivery of LY into selected individual a7r5 vascular smooth muscle cells solely via diffusion through the hydrogel plug in its tip. We demonstrate that DMB delivery is continuous, its rate can be adjusted over a broad range, and that the concentration of the dye in the cells can be determined without calibration. We also demonstrate the practicality of the DMB for studying gap junctions using clusters of connected a7r5 cells. Cells remain viable up to at least an hour during delivery experiments.

EXPERIMENTAL

All chemicals were of analytical grade. Lucifer Yellow CH potassium salt, LY, was purchased from Molecular Probes, Inc (Eugene, OR). Vascular smooth muscle cells (a7r5) were obtained from the American Tissue Culture Collection (Manassas, VI) and were grown in Dulbecco's Modified Eagle's Medium medium supplemented with fetal calf serum (10%) and antibiotics. The cells were maintained in a 37 °C incubator with 5% CO₂. Cells used for characterizing the intracellular delivery of LY were placed in Hank's Balanced Salt Solution (HBSS) during experiments conducted at room temperature.

For reagent delivery into single cells, a Diffusional Microburet, DMB, was fabricated from a glass capillary micropipette with a miniature diffusion membrane (1% agar gel) in its tip (Figure 1) according to earlier procedures¹. A Brown-Flaming micropipette puller (Model P-77) was used to pull a micropipette with ~1.5 μm i.d. The DMB body was then back filled³ with LY dissolved in intracellular buffer (in mM: 124 KCl, 14 CsCl, 9 HEPES, 9 EGTA, 0.5 CaCl₂, 5 glucose, 9 tetraethylammonium-Cl, 3 MgCl₂, 5 Na₂ATP) using a Microfill (nonmetallic thin syringe for filling micropipettes, World Precision Instruments, Inc., Sarasota, FL).

Diffusional delivery of LY was monitored with fluorescence microscopy (Zeiss Universal with epi-fluorescence) with excitation at 440 nm and the emission detected at 535 nm. For testing

the dosing of LY into individual cells (Figure 1) sparsely plated, non-touching cells ($> 150 \mu\text{m}$ between nearest cells) on a cover slip under HBSS buffer were used. The experiments were conducted at room temperature on the stage of the microscope at 40X. A DMB was moved close ($\sim 1 \mu\text{m}$) to the selected target cell and fluorescence image acquisition was started. The tip of the DMB was then inserted into the cytosol (at $\sim 2 \mu\text{m}$ depth) of the cell by poking through the plasma membrane using a 3-axis fine hydraulic micromanipulator (Narishige, MO-203). For insertion of the DMB an area close ($\sim 3 \mu\text{m}$) to the nucleus was chosen but care was taken to avoid penetration of the nucleus. For delivery into cells connected with gap junctions more densely plated cells were used after connected clusters of 2-10 cells have developed. Cell viability was tested with Trypan blue¹⁰ at the end of the experiments.

Data evaluation involved image analysis to obtain integrated fluorescence intensity for the studied cells versus time. Images were acquired using an integration time of 0.4 s for each time point. The region of the studied cell that excluded the DMB was selected. Sampling rates were chosen such that sufficient temporal resolution was achieved to map the entire delivery process that occurred over minutes. Photobleaching during the experiments was negligible. This was tested in analogous experiments involving microscopic droplets preloaded with LY, as described in reference ⁴.

RESULTS

A DMB containing a microscopic agar gel plug in its tip was back filled with 2 mM LY dissolved in intracellular buffer. The DMB was then positioned close to an a7r5 single cell that was not connected to other cells (not shown) and fluorescence image acquisition (535 nm) was started. Moving the DMB tip into the cytosol, as schematically depicted in Figure 1 resulted in an immediate rise in fluorescence in the cell. Fluorescence intensity measured in the cell *versus* time is plotted in Figure 2. Background fluorescence before impaling the cell is flat. The intracellular fluorescence level then kept increasing over many minutes. The overall trend very closely resembles a saturating exponential ($r^2 = 0.9997$) with a time constant of $\sim 2\frac{1}{2}$ min. A plateau is reached after about twelve minutes of delivery. The results shown are representative of 9 experiments.

Figure 3 shows fluorescence images obtained in another experiment when a DMB was inserted into a cell that was part of a cluster of several cells connected with gap junctions. Before impaling the selected target cell only the DMB is visible (Figure 3A). After impalement fluorescence intensity in this cell began to rise immediately. This was followed by fluorescence increase in the other cells in the cluster. These cells responded with variable delays and temporal patterns in intensity (not shown). An image taken of the cluster ~ 15 min after impalement of the target cell (Figure 3B) clearly shows that the primary cell fluoresces with the greatest intensity but other cells in the cluster have also incorporated dye molecules in detectable amounts. The sequence according to which the individual cells began to fluoresce and their emerging connection patterns indicate which cells are secondary (labeled 2), i.e., directly connected to the primary cell (labeled 1) and tertiary (label 3) connected to the primary cell through a secondary cell.

In a third experiment involving a cluster of only two connected cells (image not shown) fluorescence in the primary cell rose again immediately after impalement. There was a short but clearly manifest delay in the trend in fluorescence seen in the secondary cell (Figure 4). Small irregular fluctuations appear to be superimposed on the main trend of increase in both cells. For the secondary cell, the overall increase in LY concentration has initially a slow phase and a faster phase after about one minute. Fluorescence intensity in both cells reaches a plateau at the end.

We note that the DMB can be pulled out of a cell after delivery such that the cell membrane re-seals in most cases. It is also noteworthy that the same DMB could be used for multiple deliveries into subsequent cells. This indicates that the surface of the diffusion plug does not get obstructed by fragments of the plasma membrane. Also, each impaled cell remained viable after each experiment, as indicated by tests using Trypan blue (not shown).

DISCUSSION

Delivery of Lucifer Yellow CH into a single isolated a7r5 cell with a Diffusional Microburet

Fluorescence intensity from the analyzed region of the cell before impalement is stable (Figure 2) and is due to background current of the camera and possibly, to a small amount of light stemming from diffuse reflectance of the fluorescence of the DMB positioned adjacent to the cell. Insertion of the DMB tip in the cytosol is immediately followed by a rise in cell fluorescence. Over time this increase gradually slows until fluorescence in the cell reaches a plateau. This is expected to occur when the driving force of diffusion across the plug in the DMB tip vanishes. This happens when the cytosolic concentration of LY reaches the LY concentration in the reagent solution in the DMB body.

Self quenching of LY may be significant over macroscopic optical paths. We found this effect to be negligible, however, in solution domains of 100 μm or less in thickness (not shown). Photobleaching of LY has not been seen in microscopic buffer droplets in similar experiments and sampling rate with the same microscope and settings⁴. Therefore, neither self quenching nor photobleaching needs to be considered when analyzing the data.

The nearly perfect exponential character of the recorded saturation process suggests that the overall rate of diffusive delivery of LY by the DMB is limited by diffusion across the diffusion plug in its tip. As a consequence the cellular compartments that are accessible to LY are perfectly mixed by intracellular diffusion during the delivery process. These circumstances are described by the following differential equation:

$$V_{\text{cell}} \frac{dc_{\text{cell}}(t)}{dt} = F_{\text{DMB}}(t) = B_{\text{DMB}} [c_{\text{DMB}} - c_{\text{cell}}(t)] \quad (1)$$

where V_{cell} is total volume of those cell compartments that LY can partition into; c_{cell} is LY concentration in these cell compartments; t is time; c_{DMB} is LY concentration in the DMB body, and F_{DMB} is the instantaneous diffusive flux of LY from the DMB to the cell. B is transport coefficient of the diffusion plug in the tip, i.e., diffusional flux for unit driving force (difference in concentration between the DMB body and cytosol). With $c_{\text{cell}}(t \leq t_0) = 0$ this equation yields:

$$c_{\text{cell}}(t) = c_{\text{DMB}} (1 - e^{B(t - t_0)/V_{\text{cell}}}) \quad (2)$$

where t_0 is the time of impalement (1.9 min in the experiment shown in Figure 2). Equation 2 explains the good exponential fit seen in Figure 2.

The delivery rate as a function of time can be obtained from Equations 1 and 2:

$$F_{\text{DMB}}(t) = B c_{\text{DMB}} e^{B(t - t_0)/V_{\text{cell}}} \quad (3)$$

Thus the delivery rate is also an exponential but one that decays with time. The time constant of the delivery process, $\tau = V_{\text{cell}} / B$, is 2.5 min for the example in Figure 2.

For a nearly cylindrical plug:

$$B = D A / d \quad (4)$$

where D is diffusion coefficient of LY in the diffusion plug, and A and d are the plug's cross sectional area and length, respectively. In the tip of a pulled micropipette the shape of the gel plug will resemble more closely to a truncated cone (see Figure 1). When such a conical plug is much longer than its radius at the truncated end, the transport coefficient can be simplified²:

$$B \approx D r \pi \tan(\theta) \quad (5)$$

Here, r is internal diameter of the pipette tip and θ is the half cone angle of the diffusion plug. For tips of o.d. $\leq 1 \mu\text{m}$ as used in this work, expression 5 is valid to a very good approximation because the diffusion plug that is created in the fabrication procedure (see Materials and Methods) is $\sim 20 \mu\text{m}$ or longer (Yi, 1994).

We note that the volume of the DMB body is very large relative to the volume of an a7r5 cell. Therefore, LY concentration in the DMB will practically remain the same during intracellular delivery. This is one of the premises of the simple theory described above. Mixing of small molecules such as LY inside a cell's cytoplasm happens rapidly due to diffusion that is an efficient mechanism of homogenization over very small volumes¹⁻⁸. Using $\sim 1.5 \times 10^{-6} \text{ cm}^2/\text{s}$ as an estimate for the diffusion coefficient of LY⁴ and the approximate formula, $x \approx \sqrt{\pi D t}$, for mean diffusion length, a small bolus of LY in a cell of a radius of $\sim 10 \mu\text{m}$ will get nearly uniformly distributed in less than 1 s. As the time scale of the entire delivery process is in the minutes range ($\tau = 2 \frac{1}{2}$ min in the experiment analyzed in Figure 2) the cell compartments that are accessible to LY can be considered homogeneous during the delivery process. The equations discussed also imply that there is a good seal around the DMB and therefore the rate of egress of LY from the cell through an eventual leak is negligible compared to the rate of delivery by the DMB (Equation 1). We have established this by measuring the access resistance of some of the DMBs. These values are in the order of 10-20 $\text{M}\Omega$ and thus, far lower than the giga-ohm level leakage resistances reported for sharp electrodes²⁶. Finally, the mass transport limiting region in the DMB is restricted to within a few micrometers of the conical diffusion plug from the pipette tip for a DMB made with the usual fabrication protocol¹. Diffusional relaxation of an eventual transient in the concentration profile over such a small distance happens on the subsecond time scale². Moreover, intracellular delivery has time constants in the order of minutes. These circumstances taken together will ensure that diffusional transport through the hydrogel plug is at quasi-steady state during the entire delivery process. This has been established in earlier work on DMB delivery into microscopic droplets² and lends support for the steady state treatment of diffusional delivery in Equation 1.

A feature of practical importance when using a DMB for continuous intracellular delivery is that it is sufficient to control the pulling of the pipette (i.e., r and θ) to obtain reproducible DMBs; filling the tip with a gel plug whose length is always the same is not necessary. This is a consequence of Equation 5².

Another important feature is that when cell fluorescence reaches saturation, the concentration of LY in the cytosol is equal to the LY level in the DMB (assuming negligible leak). Thus, fluorescence intensity measured in the cell at the end of the experiment corresponds to the concentration of LY in the DMB, which is known. Consequently, the intracellular concentration of the delivered molecule can be obtained calibration free for the entire delivery process. It is noted that the measured final intensity may not directly correspond to the LY level in the DMB where binding of LY could be possible. As no LY binding sites have been reported in the literature in a7r5 cells, it is likely that LY is evenly distributed in the cytosol and eventual other compartments that are accessible to LY.

Similar to the example shown in Figure 2, a nearly perfect exponential saturation has been observed in the experiments on which we are reporting here. This implies that the effective cross section of the DMB tip through which the LY molecules diffuse into the cytosol does not vary during the process which means that the tip does not get obstructed by debris. This observation renders the DMB a fully quantitative tool for continuous intracellular delivery.

It is noted that we have obtained similar results earlier using aqueous microdroplets described in reference ² that includes a more detailed analysis of the delivery process using a DMB.

Propagation of LY delivered with a DMB among connected a7r5 cells

Fluorescent dye molecules are frequently used for studying gap junctions between connected cells²²⁻²⁵. LY is an inert dye that does not permeate the cell membrane and therefore it can partition from one cell to another only through direct connection between the cytoplasms of two cells via gap junctions. LY has been used in the past to study such junctions between connected a7r5 cells^{22, 23}. In these studies a bolus of LY was injected into the primary cell and propagation of fluorescence into the secondary cell was monitored. Another method has been perfusion of the primary cell using a patch pipette^{24, 25}. Sustained perfusion has the advantage that dynamic changes, both spontaneous and evoked, in junction conductivity can be observed. We demonstrate in this work that a DMB can also be used to deliver LY into the primary cell continuously. However, the hydrogel plug in the tip can reduce washout of biomolecules as compared to a whole cell patch by virtue of reduced diffusion of small molecules and complete blockage of the diffusional loss of molecules larger than the cutoff pore size of the hydrogel, as well as by impeding any convective exchange. Therefore, longer observations in the order of an hour are made possible. Thus, it is feasible to address larger connected cell clusters comprising of 5-10 cells.

Such an experiment is illustrated in Figure 3 where a small cluster of connected cells is studied. The fluorescence image in Figure 3A was taken after the DMB was positioned near the target, i.e., primary cell. The DMB tip is bright but none of the cells are visible in this image. After impalement, the first cell immediately began to fluoresce (not shown). Over time (minutes) directly connected, i.e., secondary cells also incorporated dye molecules. Within ~15 minutes even tertiary cells have had LY molecules partitioned in them (Figure 3B). There were observable delays between individual cells in terms of when they began to be discernible relative to the background. Based on these delays and the emerging connection patterns in the cluster secondary and tertiary cells can be distinguished, as indicated with the assigned numbers in panel B.

The dynamics of partitioning of LY from a primary into a secondary cell is shown in Figure 4, using an example of another cluster that consisted of only two connected cells. The time courses for the two cells are clearly very different. The intracellular concentration in the primary cell increases roughly as an exponential leading to saturation in 3-4 minutes. However, small fluctuations appear to be superimposed on a hypothetical “clean” exponential. A possible explanation is that the flux through the gap junctions to the adjacent secondary cells is not

stable. Knowing that delivery into just the primary cell alone would result in a “smooth” exponential, the results in the figure may indicate that most of the junctions are fully open only ~1 minute after impaling the primary cell, causing a faster increase in intracellular concentration in the secondary cell in the following period. A more detailed comparison between the two simultaneous recordings show that every temporary slow-down in the rise pattern of intensity in the primary cell tends to occur in synchrony with a faster increase in fluorescence recorded in the secondary cell. A plausible explanation can be that during these periods of slower increase in the primary cell DMB delivery is mostly compensating for “loss” of LY via gap junctions to the secondary cell. These observations infer that permeability of the junctions may undergo variations during the early part of the delivery experiment.

These results demonstrate that DMB delivery into a cell within a cluster can be used to study dynamic characteristics of gap junctions, such as variations in throughput as a function of time. Besides such qualitative information, a quantitative assessment of junction activity can also be derived from the results of such experiments performed with the DMB, as explained here for only two connected cells. The final plateau levels in measured fluorescence indicate that the cytosolic concentration of LY has reached in both cells the LY level in the DMB. From this information the absolute concentrations in both cells can be obtained as functions of time. Thus concentrations on both sides of the junctions are available and can be correlated with the corresponding states of the gap junctions observed in the recordings. The absolute flux of dye delivery into the primary cell can also be calculated from the intracellular concentration in the impaled cell using Equation 1 if the transport coefficient, B , of the DMB is known from precalibration performed in a droplet of known volume, as described in reference ². From the integral of this flux the total amount of dye that has passed through the junctions in any given time period into the secondary cell can be derived, provided that the ratio of the volumes of the two cells is made available. This information can be estimated using total fluorescence intensities of the two cells, or obtained quantitatively if a z-stack of the cells is acquired in the confocal mode of the microscope at the end of the experiment. Either estimate can be used for estimating the ratio of LY-accessible volumes of the two cells.

We note that the volumes estimated with either method will reflect the sum of those intracellular compartments into which LY partitions. However, it is this volume fraction of the cells that is *de facto* required to characterize gap junction activity. It is also to be noted that the plateau fluorescence measured at the end of the delivery is typically different for the two connected cells despite that the concentrations are equal after saturation, such as in Figure 4. This is because the recorded intensity contains information also on cell morphology, size, area assessed, and background fluorescence.

CONCLUSIONS

Delivering dyes and other molecules directly into the cytosol of live cells has importance in many areas of biological and clinical research. Delivery of boluses and sustained perfusion of single cells are both feasible with a number of available methodologies.

We described in this work the use of a Diffusional Microburet for continuous delivery of molecules into the cytosol. The DMB technique has been analyzed and explored earlier for the delivery of substances into microscopic aqueous droplets for different types of application. These results helped adapt the DMB for intracellular delivery of molecules in this work.

The use of the DMB for cellular studies has several benefits that are complementary with other approaches. Some of these benefits are: continuous delivery whose rate is adjustable and quantifiable; negligible dialysis of cytosolic molecules through the tip of the DMB; delivery that can last up to at least an hour without apparent damage to the target cell; calibration-free

assessment of the instantaneous cytosolic concentration of the delivered molecule; quantitative estimation of transport between connected cells across gap junctions; and the ability of delivering any type of molecule into selected target cells. These features rest upon the hydrogel plug in the pipette tip whose pore distribution and effective cross sectional area remain unchanged during the experiment. Different methodologies of analytical chemistry that require precise reagent delivery into samples can be adapted to studies on single live cells using this technology.

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Intracellular dye delivery with a DMB

Figure 1.

Schematics of the experimental arrangement for the delivery of molecules of interest into a target cell with a Diffusional Microburet, DMB. The DMB is made of a pulled glass capillary with a submicron tip that is filled with a microscopic hydrogel plug. LY diffuses from the DMB body through the plug into the cytosol driven by concentration gradient. This results in continuous dosing of the cell but without the addition of volume to the cytosol. The delivery process is reproducible for the same DMB. Flux can be adjusted by proper choice of tip diameter, type of hydrogel and its pore size, and concentration of the molecule of interest in the DMB body.

Figure 2.

Time course of the delivery of a fluorescent dye, Lucifer Yellow CH, LY, into a single a7r5 vascular smooth muscle cell. Fluorescence intensity was obtained by averaging over a $25 \mu\text{m}^2$ area in the cell that excluded the nucleus and the DMB tip. Delivery began by moving the tip of a DMB filled with 2 mM LY in intracellular buffer into the cytosol at $t = t_0$. Intensity values measured before impalement: ○; intensity after impalement: ●; exponential least-squares fit to the data obtained after impalement: solid line.

Figure 3.

Fluorescence images of a cluster of a7r5 cells connected with gap junctions before impalement with a DMB (Panel A), and 15 minutes thereafter (Panel B). In panel A only the DMB tip is visible that is loaded with LY molecules (2 mM in intracellular buffer). In Panel B a number of cells are also clearly discernible due to incorporation of LY. LY first diffused from the DMB into the impaled primary cell (1). It then propagated into several secondary cells (2) that are directly connected to the primary cell. Some tertiary cells (3) that are connected to the secondary cells but have no direct connection to the primary cell also began to fluoresce by the time the image in Panel B was taken. The cone angle of the DMB tip appears to be much larger in the images than what it actually is. This is because the DMB's axis is slightly slanted relative to the vertical axis of the microscope, as indicated in Figure 1.

Figure 4.

Time course of the delivery of a fluorescent dye, Lucifer Yellow CH, LY, into a single a7r5 vascular smooth muscle cell connected to a secondary cell via gap junctions. Fluorescence intensity was obtained by averaging over a $25 \mu\text{m}^2$ area in the cells, excluding the nucleus in both cells and the DMB tip in the primary cell. Delivery began by moving the tip of the DMB into the cytosol of the primary cell as indicated by the arrow. Intensity values measured for the primary cell before impalement: $\square$; intensity after impalement: $\blacksquare$; same for the secondary cell: ∇ , and $\blacktriangledown$, respectively.