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A picoliter 'fountain-pen' using co-axial dual pipettes

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Abstract

A double pipette system for local, controlled drug infusion is presented. Two concentric pipettes can be manipulated separately and pressurized independently by a designated double holder. The inner pipette is loaded with the desirable solution (drug), and functions as a source, while the outer one is a sink. This gives a flow of the solution between the two pipettes that protrudes only a small distance into the surrounding fluid and does not diffuse away. Time resolution of the infusion is highly controllable, and oscillatory flow can be generated. Three implementations of the double pipette system are demonstrated. We show that local application of neurotransmitters in neuronal networks is an efficient way of stimulating activity in the network. We then present a wet micro lithography technique using topical application of proteins onto the substrate. Finally, we show that we can localize a given drug on a small targeted part of a cell.

Keywords: Micropipettes; Local perfusion; Chemical stimulation; Oscillatory stimulation; Wet lithography; P19 EC stem cells

1. Introduction

The ability to localize chemical and biological functions to the micrometer scale is at the heart of many recent and revolutionary advances in the life sciences. Examples come from a multitude of emergent technologies ranging from DNA (Schena et al., 1995) and protein (MacBeath and Schreiber, 2000) arrays, through microfluidics (Beebe et al., 2002; Chovan and Guttman, 2002) and microlithography in dry (Delamarche et al., 1997; Piner et al., 1999; Glezos et al., 2002) and physiological (Shivshankar and Libchaber, 1998; Bruckbauer et al., 2002) conditions, and all the way to single cell stimulation (Dodd et al., 1981; Aston-Jones et al., 1991; Cattaert et al., 1994; Marchand and Pearlstein, 1995). However, the ability to apply minute amounts of fluid at these typical sizes both topically and precisely, for well defined times and without leakage, is still in its infancy.

In this paper we present a novel method for highly controlled injection of a minute volume of one fluid (on the order of 10 μm^3) into another fluid. Two concentric pipettes are brought into proximity of the target, and fluid that is ejected from the inner one protrudes only slightly into the environment, just enough to touch the target before it is entrained in a flow going from the surrounding fluid into the external pipette. The extent that the jet penetrates into the surroundings and its duration, are the two parameters that determine the effect of the internal fluid, and can be precisely controlled over a wide range of values. The apparatus and jet are depicted in Fig. 1.

In conventional micropipette injection methods, the size and shape of the injection area are highly affected by a multitude of factors like pressures, pipette tip radius and shape and liquid flows in the bath (Hanani, 1997) making them hard to control. In contrast, here the jet can be precisely tuned in sizes between a few micrometers and a few hundred micrometers, and given in pulse duration times that range from tens of milliseconds to a few minutes. The leakage from the jet to the surrounding fluid is extremely small, allowing long injection times with no danger of affecting anything but the target.

In the following, we first characterize the properties of the new system. We calibrate the flow velocities, and

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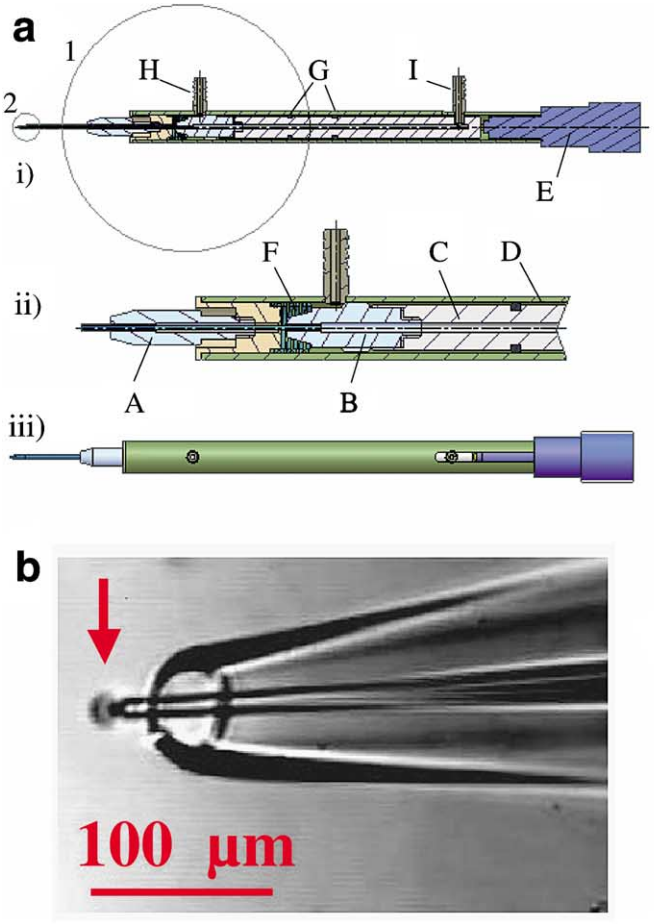

Fig. 1

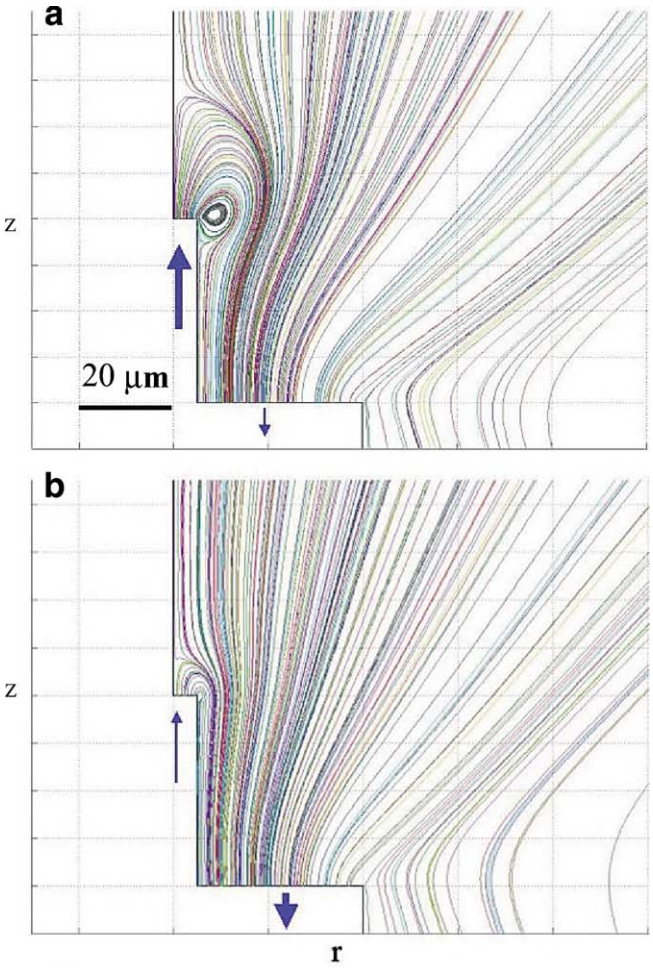

Fig. 2

Fig. 1. The concentric pipettes. (a) A schematic of the double pipette system. The two pipettes are inserted into separate capillary grip heads (A,B-Eppendorf catalog #5242022.000 of which the inner one B was slightly modified to allow aligned insertion into D) and fit unto two tubular holders (C,D), whose relative position is controlled by a DC motor (E) and a spring (F). The two holders are aligned by two O-rings (G), which also isolate between two airtight chambers with separate pressure ports (H, I). (ii) Is a blow up of region 1 in (i), (iii) is an external view of the holder. (b) A microscope image at the area designated 2 in (a). Outflow from inner pipette attracted by the suction of the outer pipette. An arrow points to the infusion jet, which appears darker than its surrounding due to the difference in the index of refraction.

Fig. 2. Simulation of the flow between the two pipettes. Flow lines are plotted in the (r, z) plane; the arrow on the left of each figure marks the symmetry axis. The inner pipette protrudes 40 μm from the outer pipette. (a) To reconstruct a large (50 μm) jet diameter with the given radii of the pipettes, the injection velocity needed is high (0.06 m/s) while the inward current is small (0.07 m/s); (b) a jet diameter of less than 10 μm is obtained with an injection velocity of 0.03 m/s and a fast collection of 0.3 m/s.

then directly measure the amount of leakage. We use numerical simulations to explore the range of possible flow parameters, and find a quantitative agreement with the results of the calibration and leakage measurement. Oscillatory perfusion at tens of Hertz is performed. We then go on to demonstrate the usage of the technique in three different situations, chosen from a multitude of possible applications.

The first application is that of *local neuronal network stimulation*. Presently neuronal excitation can be achieved using electric stimulation either intra- or extra-cellulary, or by adding drugs (neuro-modulators) either to the entire media or at a specific point by iontophorosis (Dodd et al., 1981; Aston-Jones et al., 1991; Bruckbauer et al., 2002) and micropressure (Aston-Jones et al., 1991; Cattaert et al., 1994; Marchand and Pearlstein, 1995) techniques. Each of these methods has its shortcomings, many of which can be resolved using our system.

The second use is that of *wet lithography*. Loading the inner electrode with any protein that will adhere to the surface, we can create patterns along the surface by moving the double pipette and its jet along it. This may even be done in samples containing live cells without disturbing them, due to the local nature of the injection. Patterns can be formed while taking into account positions of cells, at a specific stage in the cell cycle, etc. We tested the accuracy of this method by fluor-

escent protein lithography, using the tools of protein chip preparation and detection.

The third field demonstrated for our system is cell biology, *applying drugs locally with sub-cellular resolution*. Typical cells are tens of micrometers across, thus, by using a jet radius of dimension less than 10 micrometers we can apply drugs to a particular section of a cell's membrane. The ability to distinguish between local and global effects on a cell opens the way to many interesting questions (Axelrod et al., 1976; Verveer et al., 2000).

2. Methods

2.1. Experimental setup

The double holder consists of two concentric single pipette holders (Fig. 1a) that center and align the two pipettes. The inner part can be slid forward for a smooth insertion of one pipette into the other (Roberts and Almers, 1984; Jonas et al., 1997). Once inserted, mounted on a micromanipulator, and brought close to their final position the two parts are locked in together with a DC motor, which will later, under the microscope, allow fine positioning of the inner pipette with respect to the outer on.

Once closed, two airtight chambers are formed inside the holder (Takeshi et al., 1988). They are separated by O-rings and the pressure in each can be controlled from the outside through appropriate ports, connected to separate pressure sources (e.g. syringes) and capacitance pressure meters (made by Validyne USA) in parallel. We typically used borosilicate pipettes that can be slipped one on the other, e.g. inner pipette with (OD = 1.0 mm, ID = 0.78 mm) and outer one with (OD = 1.5 mm, ID = 1.1 mm). The pipettes are pulled with a Sutter P-2000 puller and the tip diameters are set in accordance with the desired jet size.

The double pipettes are calibrated prior to each experiment in a procedure that lasts one minute and allows easy control of the injection area's size and shape. When injecting a solution (e.g. PBS) with different index of refraction than its surrounding fluid (e.g. water) the jet can be visualized easily and clearly (Fig. 1b). Direct visualization of the infusion area is more difficult when the index of refraction of the medium is closer to that of the infused solution, as is often the case. Reproducing the pressures calibrated with a high difference of refraction indices will then reproduce the desired perfusion area.

2.2. Flow velocities

The pressures applied to the two pipettes are crucial parameters that define the size of the infusion area. Pressures (measured as a differential from atmospheric pressure) ranging from +20 to +60 hPA are applied to the inner pipette and a suction of −10 to −45 hPa are applied to the outer pipette. The velocities of the in- and out-going currents are measured by the change in liquid volume inside each pipette while the other pipette was kept at atmospheric pressure, measured over about a minute.

The measured velocities into an outer pipette with a 40 μm radius range from 0.07 m/s at an applied pressure of −10 hPA, all the way up to 0.3 m/s at −45 hPA. The velocity at which the applied fluid exits an inner pipette with a radius of 5 μm rose from 0.03 m/s at +20 hPa to 0.06 m/s at +60 hPa.

2.3. Lithograpy

Aldehyde coated slides (Telechem International, SuperAldehyde Substrates) were used. In some cases the slides were additionally coated with BSA-biotin (Sigma) that attached covalently to the aldehyde groups on the slide.

For infusion a pipette was loaded with Cy5-conjugated Streptavidin (Amersham Pharmacia Biotech) diluted in PBS to a concentration of 17 nM. Infusion in all cases was performed in a PBS environment either on the aldehyde-coated slides or on the aldehyde slides that were coated with BSA-biotin.

Fluorescence of the Cy5-probed slides were scanned by a Packard ScanArray 4000 scanner at a 5 μm resolution. Relative intensity of the spots was measured and compared to relative intensities produced earlier on the same slides by Cy5-conjugated Streptavidin in PBS at known concentrations.

2.4. Cell culture

Undifferentiated P19 cells were propagated in minimum essential medium alpha MEM supplemented with 5% fetal calf serum. Neuronal differentiation was done using standard procedures (Yao et al., 1995). Neurons were plated onto laminin and fibronectin treated glass coverslips. Using micro-lithography, laminin and fibronection were restricted to one dimensional lines, producing linear neural structures. On the 3rd day after plating, Cytosinearabinoside (Ara-C) was added at a final concentration of 10 μM to inhibit division of non-differentiated cells. After 2 more days the cells were switched to neurobasal medium containing NB supplement (both by GIBCO). Fluorescent measurements on the cells were performed about 21 days post plating.

2.5. Coverslip patterning

Glass coverslips are patterned to make only specific locations (usually lines) available for cell adhesion. This

is done in two steps: first the entire coverslip is coated to make it inert to protein and thus cell adhesion. Then, lines are scratched through this coating and the entire coverslip is immersed in a solution of adhesion proteins, these attach to the lines only, giving us the desired result.

The inert coating is actually composed of four layers:

The first two layers are evaporated on the glass, 40 Å layer of gold on 8 Å of chromium. The coverslips were further coated with octadecanthiol. The molecules of octadecanthiol are linear and highly hydrophobic with a high affinity for gold on one end, causing them to self assemble into a hydrophopbic monolayer (Prime and Whitesides, 1991). For the fourth and last layer, coverslips are immersed in a solution of Pluronics F108 Prill (BASF). Pluronics molecules are long linear polymers composed of three parts, roughly equal in length, the middle part is hydrophobic, the other two hydrophilic. The hydrophobic part attaches to the hydrophobic surface of the coverslip, while the hydrophilic parts are left to wiggle around in the media over the surface (Amiji and Park, 1992).

Lines are scratched onto the surface with a sharp blade, cutting through the layers that cover the glass, and exposing it. In this method patterns can be drawn on the cover slip. The cover slip is further immersed in a solution of fibronectin and laminin. Due to the entropic repulsion of the ends of the Pluronics molecules this layer adheres specifically to the previously exposed lines.

The differentiated P19 cells are plated on these treated coverslips, adhering to the exposed lines only. Neuronal aggregate size depends on the width of the line, but lines that are not thick enough to support small aggregates will remain clean of neurons. An optimal line width of about 100 micrometers is used. The repulsive force of the Pluronics coating does not fade in time, and despite the secretion of adhesion proteins by the cells themselves, the cells remain exclusively on the lines throughout their life.

2.6. Imaging neural activity by fluorescent imaging

Cells were incubated for 30 min in a balanced salt solution in the presence of 10 μM cell-permeant Oregon Green Bapta-1 (Moleculer Probes) and 0.1% of Pluronics F127 Prill (BASF). Cells were then washed and placed in Tyrode's Solution. Cells were mounted on a Zeiss Axiovert 135TV microscope, kept at 37 °C, and photographed through a 10× lens by a Hamamtsu C2400-87 CCD camera fitted with a 0.5× adapter, the images were stored on video and analyzed off line.

2.7. Asymmetric extra-cellular drug application

The inner pipette was loaded with Oregon Green conjugated concanavalin A (Molecular Probes), a protein that attaches to sugars on the lipid membrane, at a concentration of 200 μg in 1 ml of PBS. Sparsely plated undifferentiated P19 cells were placed in Tyrode's solution, mounted on the microscope and maintained at 37 °C. Fluorescent images were gathered once every minute using a 100× lens.

3. Results and discussion

3.1. Leak detection

We monitored the leakage from the double pipette by measuring the amount of a fluorescent marker in the surrounding fluid. The inner electrode was loaded with 6×10^{-3} M of the fluorescent marker TAMRA (6-carboxytetramethyl-rhodamine) in PBS, and the double pipette immersed in 1 ml of water. The measurements were divided into three experiments with different suction pressures (measured as a differential from atmospheric pressure): no suction pressure—the infusion jets out, moderate pressure (−20 hPa)—producing an infusion diameter of around 40 μm, and high pressure (−45 hPa)—producing a 5 μm jet. After each experiment the water was collected and monitored. The experiments were repeated for several infusion times.

TAMRA concentrations were then measured using a homemade fluorimeter sensitive to 50 pM. The ratio between the concentrations in experiments where both outflow and suction were applied to those in which there was only outflow gave us the percent of the total flow from the inner pipette that leaked into the surroundings.

In the sets of measurements where no suction was applied, the TAMRA concentration scaled with time. Calculating the infusion velocity by these concentrations gave us numbers ranging between 1 and 10 cm/s, in agreement with our earlier measurements (see Section 2.1).

In the sets of measurements where moderate suction pressure was applied, the concentration detected was more than three orders of magnitude lower than those detected in the experiments that used free injection. These measurements did not scale with time. This leads us to conclude that the losses from the pipette occur mostly right after it was immersed in the water but before suction was applied. In real applications these losses are often negligible, because they occur away from the actual infusion area, and disperse over the entire sample.

In the measurements where higher pressure was applied, again there was no scaling with time. This time the measured TAMRA concentrations were more than four orders of magnitude lower than those of free injection. This is probably because the higher suction retrieves some of the material lost while immersing the pipette.

We conclude that the leakage of the double pipette is at most 0.1%, but probably even better.

3.2. Numerical simulations

Once the flow velocities in the perfusion jet are measured (see Section 2.1), we can estimate the system's Reynolds number:

$$\mathrm{Re} = \frac{d \cdot v \cdot \rho}{\eta}.$$

Plugging in $\rho = 10$ kg/m^3 the density of water, $\eta = 10^{-3}$ kg/ms their dynamic viscosity, $d = 10$–100 μm as a typical scale, and $v = 0.1$ m/s as a typical velocity we get a Reynolds number of 1–10. The system is neither laminar nor turbulent making analytic calculations difficult.

Using a designated MATLAB program, we solved the cylindrically symmetric Navier–Stokes equation numerically, using the pipette system and the inwards and outward liquid velocities that were measured earlier as the boundary and boundary conditions. In Fig. 2, two flow diagrams, projected on the (r, z) plane, are plotted. The full range of available jet diameters scales with pipette radii and with the boundaries given in Fig. 2, is $\sim$2–100 micrometers.

In both cases the infusion current enters the external pipette tens of micrometers away from its edge, a fact also observed in the experimental imaging of the flow. To have a diffusive leak out of the system, drug molecules would then have to diffuse over this length during the time they are flowing between the pipettes. The importance of diffusion is given by the Peclet number, a dimensionless ratio of the time scales of diffusion and flow:

$$\mathrm{Pe} = \frac{R \cdot v}{D},$$

R is the outer pipette radius, v the flow velocity, and $D \approx 10^{-9}$–10^{-10} m^2/s the diffusion constant. Plugging in the relevant values, we get $\mathrm{Pe} \approx 10^2$–10^3, which means that the role of diffusion in this system is negligible.

3.3. Oscillatory perfusion

By modulating the pressure source for the pipette using mechanical means, oscillatory pulses at frequencies on the order of tens of Hertz are easily obtained. For a single pipette system this would only add the accumulating perfused substance at a specific frequency. In contrast, the double pipette system allows a totally different approach whence the concentration around the pipette changes from nearly zero to a specific desired concentration at the oscillation frequency. This may be useful to chemically stimulate neurons, which react more strongly to repeated bursts than to a single stimulation, or cardiac cells that respond strongly at frequencies close to their beating frequency.

To estimate the maximal frequencies that may be used we calculate lower bounds on the jet formation time:

- The time it takes for a perfused molecule to travel from the inner pipette into the larger one. We obtain an estimate of this time by dividing the distance d a molecule travels by its velocity v: $T_1 = d/v$. d is the jet dimension and v the perfusion velocity.
- The viscosity of the fluid limits the rate at which a velocity can be built up from zero. To estimate this, we must keep in mind that the liquid velocity across the diameter of the pipette is not constant: on the layer closest to the pipette boundary it is zero, while maximum velocity is achieved on the center of pipette. The relative motion of adjacent layers results in friction between them (viscosity) which opposes the flow and sets another time limit: $T_2 = r^2/v$. v is the fluid viscosity and r the inner tip diameter.
- The time it takes pressure to travel along the piping to the pipette. $T_3 = L/V_s$. L is the length and V_s the speed of sound.

All are estimated at 1 ms or less, suggesting that perfusion frequencies up to hundreds of Hertz are possible.

We tested the system at frequencies up to 25 Hz, at which our video observation could verify the effectiveness of the modulation. The inner pipette was connected to a syringe hooked up to a rotation motor. During one cycle of the motor the pressure on the inner pipette changes between atmospheric pressure and the calibrated pressure. The pressure on the outer pipette was held at a constant suction pressure. We set the motor to 25 RPM and through the microscope, recorded the perfusion jet at video rate (50 Hz). During a few minutes of such recording, we could find parts in which there is a zero phase difference between the video and the oscillating jet, so that the full perfusion jet is visible on every second frame while no jet is seen on the others. Consecutive video images of a 10 Hz oscillatory perfusion are shown in Fig. 3.

3.4. Local neuronal network stimulation

To construct a neuronal network, we used the P19 cell line (Finley et al., 1996). This is in contrast to most current approaches that prefer using a primary cell culture for constructing a neuronal network. The advantages of a cell line are manifold, starting from the ease and simplicity of cultures to repeatability and reliability along time, as well as amenability to genetic manipulation. The P19 cell line is known to create networks with spontaneous activity, and express both

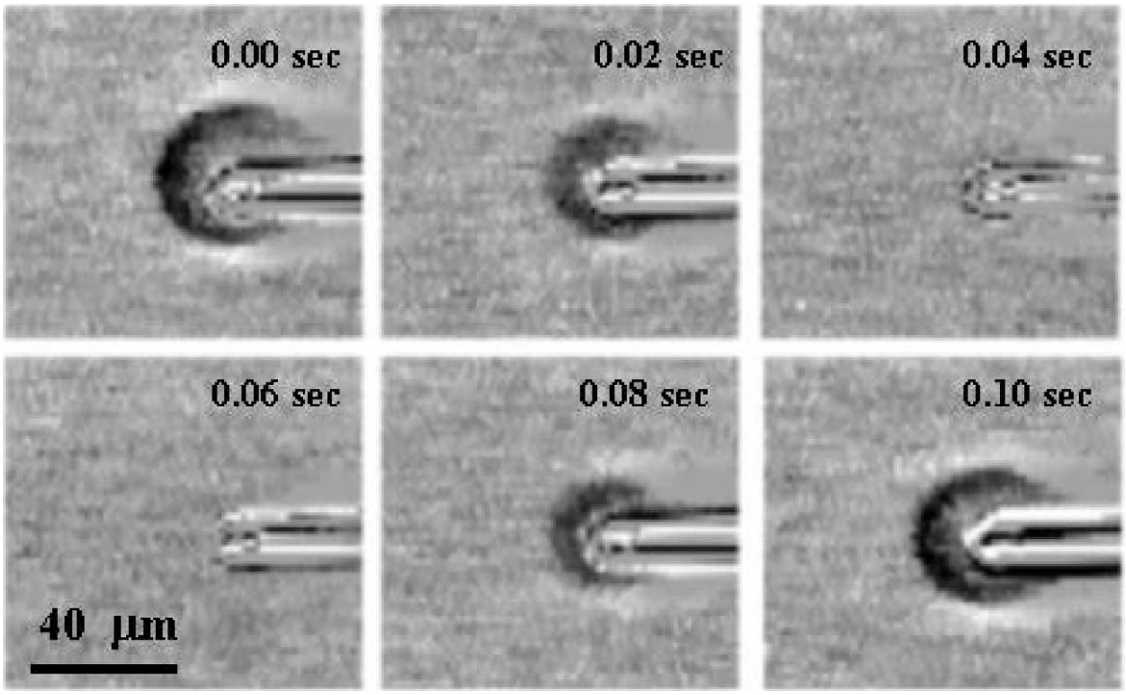

Fig. 3. Six consecutive video frames showing a full cycle of a 10 Hz oscillatory perfusion. Perfusing periodically through a single pipette system would only add the accumulating substance at a specific frequency. In contrast, the double pipette system allows a totally different approach in which the presence of the perfused material around the pipette tip is truly periodic.

excitatory and inhibitory synapses (Finley et al., 1996). Cells were differentiated into neurons and plated unto biologically inert glass slides, patterned by 100 micrometer thick lines to which neurons may adhere. Once plated, neurons migrate on these lines and bundle into aggregates of, typically, around 100 neurons. The distance between neighbouring aggregates is hundreds of micrometers, and is spanned by thick neurite fascicles that interconnect them. The result is a linearly connected structure, whose main nodes are composed of many neurons with multiple intraconnections, comprising what is known as a 'layered network'. Neurons are maintained in culture for approximately 3 weeks.

The cells were loaded with a calcium sensitive fluorescent dye, to monitor neuron activity. Two concentric pipettes were adjusted to make an infusion diameter of ~ 100 μm, in accordance with the aggregate size (smaller infusion areas were less likely to produce a response). The inner pipette was filled with extra-cellular solution enriched by 200 μM glutamate (an excitatory neurotransmitter).

Three experiments were conducted in succession. In the first experiment a 30 s local injection was performed over an aggregate (see Fig. 4a). Fluorescent images from a Calcium sensitive dye (Fig. 4b) were collected every 40 ms. A significant rise in the fluorescent illumination of the aggregate under the pipette, as well as neighboring aggregates was observed once the infusion had begun (see Fig. 4c).

In a control experiment, the injection was performed 300 micrometers away from the aggregate (Fig. 4d). This time the infusion did not induce a rise in fluorescence in any of the aggregates (see Fig. 4e). Note, that although the pipette was closer than it was in the previous experiment to aggregates 2 and 5, the infusion area did not overlap them, and thus did not affect them.

The third experiment repeated the first, and produced similar results, proving the neurons can be activated more than once.

Two conclusions can be made:

- The infusion area was local (and at the desired scale).
- The rise of fluorescence in aggregates 2–6 was caused by the neural activity initiated in the net.

Several interesting details arise from Fig. 4b. First, the transient of 3 s between the initiation of application of the glutamate signal and the appearance of a Calcium

Fig. 4. The inner pipette was filled with Glutamate enriched medium and infused at several locations around a neural net. The neurons are loaded with calcium sensitive fluorescent dye. (a) Local perfusion is performed just above aggregate 1. (b) A fluorescent image of the same area. (c) The response of aggregates 1–6 (as marked in (a)) to the perfusion. The perfusion was initiated manually, at $t = 0$ and takes 1–2 s to reach full pressure. The relative 'y' positions of the graphs are arbitrary. Aggregates which were not plotted showed similar behavior. (d) In this control experiment the perfusion area is moved so that it does not overlap any of the aggregates. (e) The response of the same aggregates to the control perfusion.

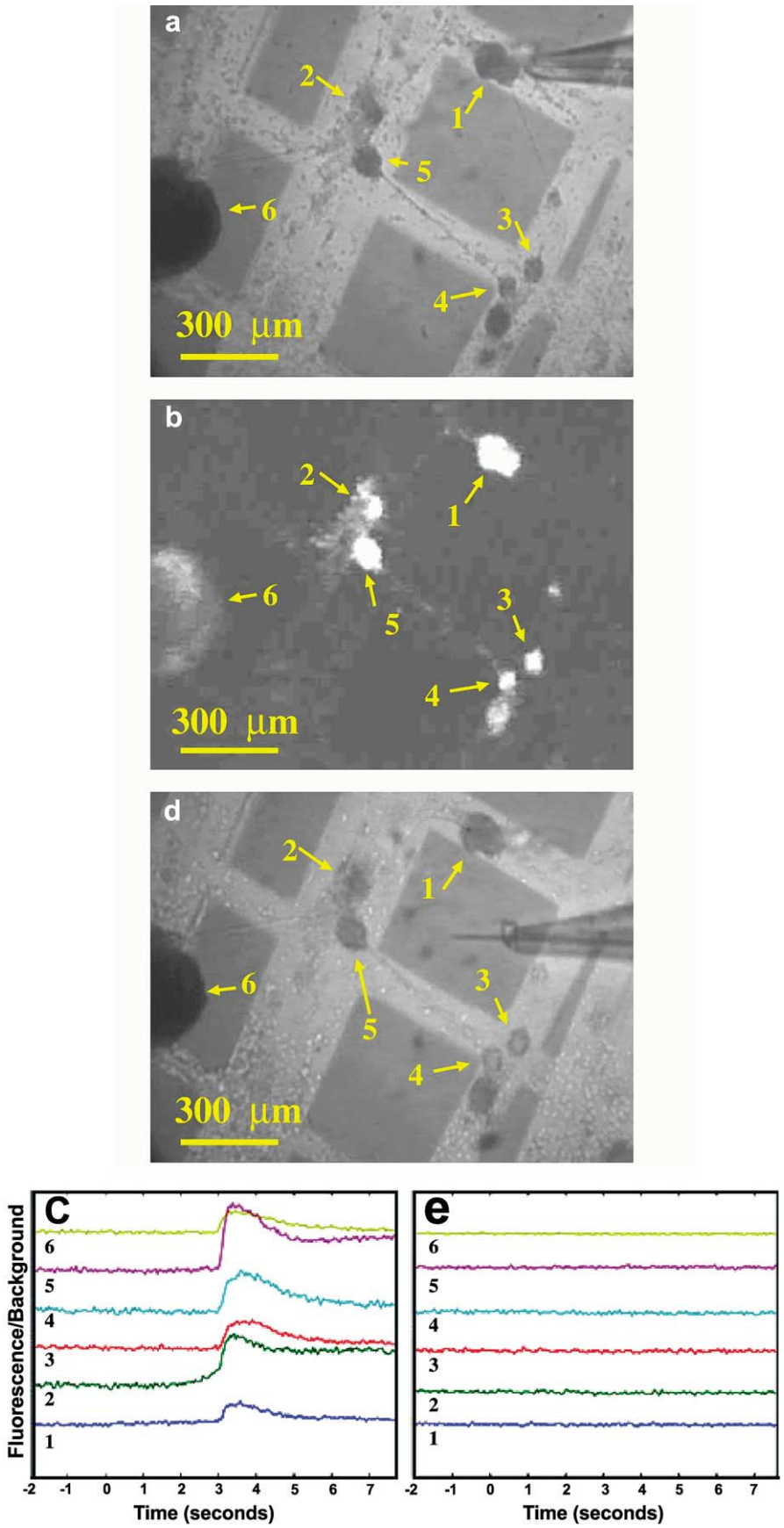
a
2
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Fig. 4

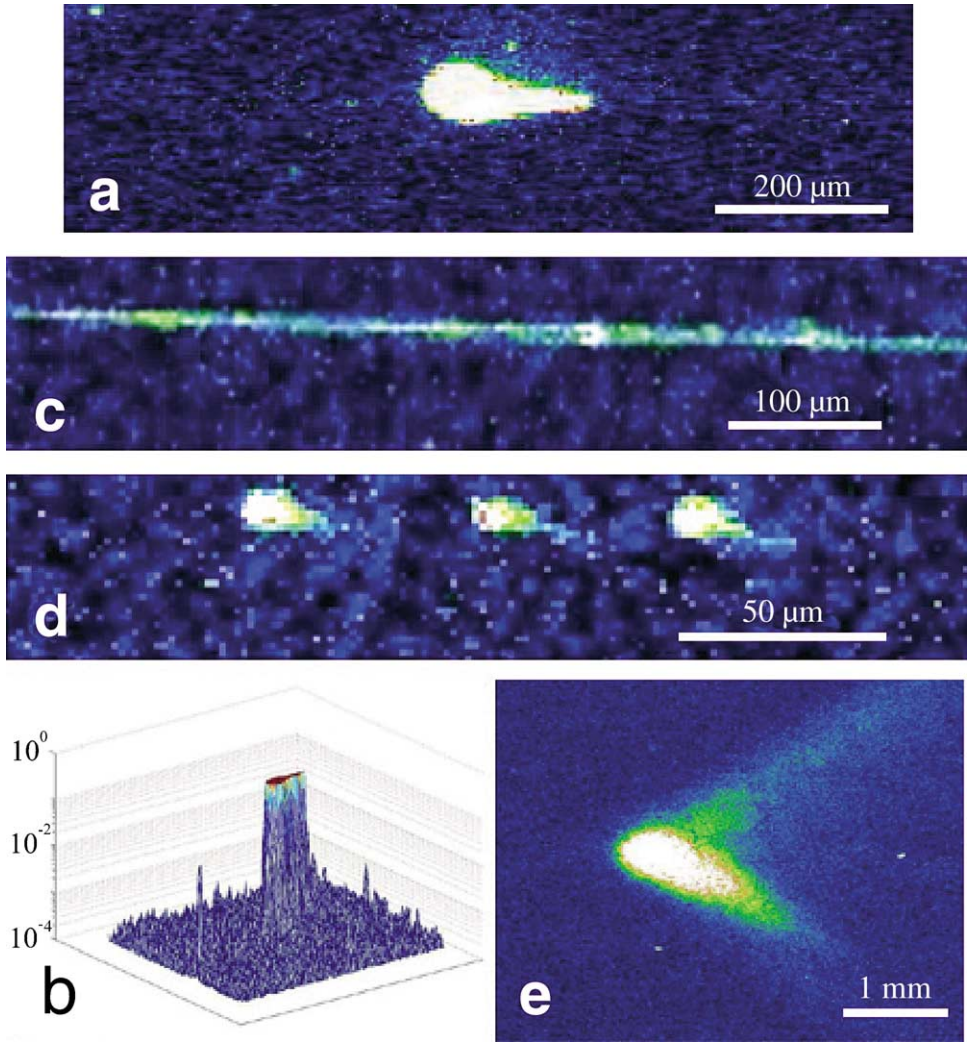

Fig. 5. Cy5 conjugated streptavidin was infused near the surface of BSA-biotin coated slides, and adsorbed to it via by either Streptavidin-Biotin or Straptavidin-aldehyde interactions. (a,c,d,e)—Packard Scannarray fluorescent scans of the infusion areas. (a) A large perfusion area, in a size typical for aggregate stimulation. The perfusion time is 3 min long. (b) A translation of figure (a) into Cy5 conjugated streptavidin concentrations relative to the concentration where the ScanArray begins to saturate. Note the sharp borders indicating low leakage. (c) A thin line about 10 μm across produced by moving the pipette across the glass while perfusing. (d) An Array of small spots 20 μm in diameter, using short perfusion times. The spots are packed in an array at a density of 400 spots per mm^2 and may provide a possible method of constructing bio-chips. As the numerical simulation shows (see Section 3.2), the spot scales linearly with system dimensions so that making even smaller spots involves using finer pipettes. (e) A control experiment where low pressure was applied to the inner pipette, and no suction to the outer pipette. Although perfusion time was only 30 s (compare to a) locality is lost (notice the scale bar) and borders are blurry.

signal indicates that the process of stimulation involves a complex process. A single puff of glutamate does not invoke a global response (which is enough to show up in the fluorescence); it takes an integrated application over more than a second to invoke such a response.

Some of this complexity becomes apparent when we notice that the strong initial rise in fluorescence occurs first not in the target aggregate 1, but rather its nearest neighbor aggregate 2. This indicates that a few neurons excited in aggregate 1 excite neurons in aggregate 2, which more efficiently excite all of aggregate 2, and only then does all of aggregate 1 along with the rest of the network get fully stimulated.

3.5. Wet microlithography

The concentric pipettes are also used for wet microlithography. Moving the double pipette and its jet towards the surface and along it creates protein patterns.

The accuracy of this method was tested by fluorescent protein lithography. We used either aldehyde or BSA-biotin coated slides. The double pipette system was loaded with streptavidin conjugated with the fluorescent marker. Streptavidin has high chemical affinity to both coatings of the slide. Infusion in all cases was performed in a PBS environment. Thus we were able to fluores-

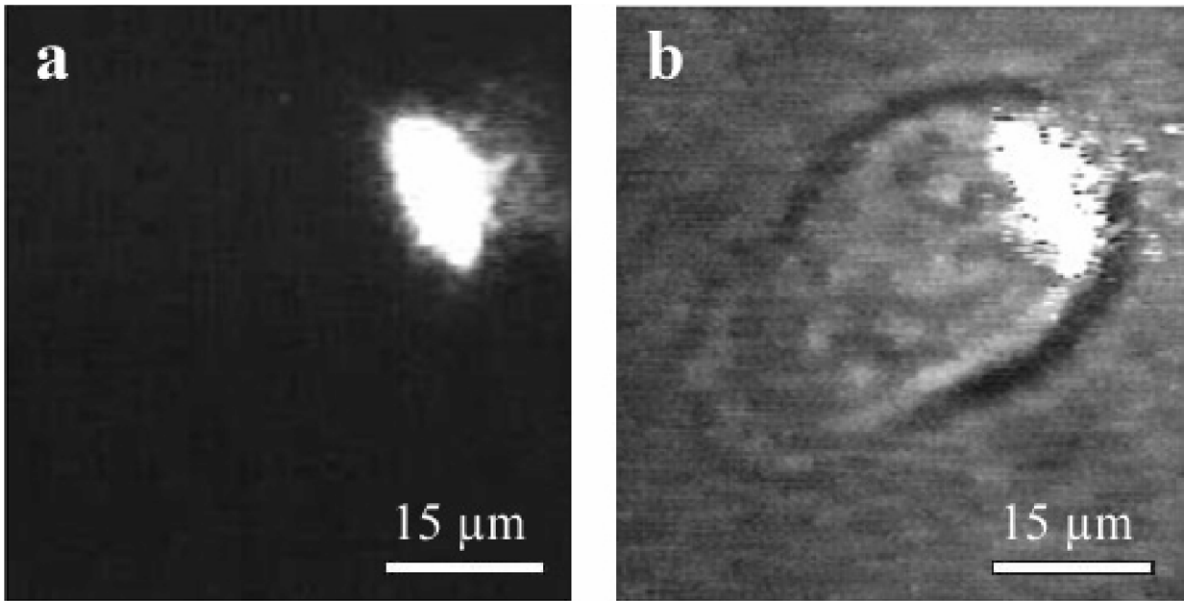

Fig. 6. Oregon Green conjugated concanavalin-A was infused near one side of a P19 cell. (a) The fluorescent image of the cell after the pipette was moved away. (b) The same image superimposed with a white light picture of the cell.

cently mark the perfusion area. We produced sharp-bordered infusion jets ranging between 200 and 2×10^4 μm^2 in size (Fig. 5). While other methods obtain somewhat smaller spot size, none offer this wide range of available spot sizes and fluids (for example, iontophorosis (Bruckbauer et al., 2002) is limited by the need to use charged molecules, and diffusion is a crucial issue there while the dip-pen method (Piner et al., 1999) requires a dry environment).

3.6. Subcellular drug application

Concanavalin-A conjugated with an Oregon Green fluorescent marker was locally applied so that the injection jet partially covered an undifferentiated P19 cell. The pipette was then moved away and fluorescent images of the cell were taken. As is shown in Fig. 6, a bright spot of around 5 μm is apparent on the side of the cell infused by the pipette while the rest of the cell remained fluorescently dark. Neighboring cells remained dark as well. Subsequent imaging of the cell showed practically no diffusion of the marker, a characteristic of Concanavalin that has previously been reported (Henis, 1984; Kucik et al., 1999).

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Corrigendum

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