Localized Functional Chemical Stimulation of TE 671 Cells Cultured on Nanoporous Membrane by Calcein and Acetylcholine

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ABSTRACT Acetylcholine sensitive TE 671 cells were cultured on nanoporous membranes and chemically stimulated by localized application of i), calcein-AM and ii), acetylcholine, respectively, onto the bottom face of the membrane employing an ink jet print head. Stimulus correlated response of cells was recorded by fluorescence microscopy with temporal and spatial resolution. Calcein fluorescence develops as a result of intracellular enzymatic conversion of calcein-AM, whereas Ca^{2+} imaging using fluo-4 dye was employed to visualize cellular response to acetylcholine stimulation. Using 25 pl droplets and substance concentration ranging from 10 μM to 1 mM on Nucleopore membranes with pore diameters between 50 nm and 1 μm, a resolution on the order of 50 μm was achieved.

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INTRODUCTION

Electrostimulation has been the method of choice for almost all neurostimulation devices in use to date. As it relies solely on membrane depolarization by electric fields to induce a cellular response, it lacks, however, the cell type selectivity and spatial resolution in principle achievable by chemical stimulation. Several microfabricated drug delivery systems for controlled release of biochemically active compounds have been proposed (1–3). Earlier attempts to use microfluidic devices for chemical stimulation of cells suffered from the problem of uncontrolled leakage from open microapertures into the medium and consequently undefined concentration conditions (4,5). In contrast, our novel ''air gap''-scheme (6) (Fig. 1) avoids such leakage completely. It facilitates the precise positioning of the application spot directly under or at a lateral distance with respect to cells of interest. Either calcein-AM or acetylcholine was used for stimulation. Concentration gradients develop along the surface of the cell layer eliciting specific cellular responses.

METHOD

Tissue chambers consisting of a glass ring with a Nucleopore membrane (Whatman International, Maidstone, UK) with pore sizes ranging from 50 nm to 1 μm glued to their bottom were homemade and treated in an air plasma (30 s) before cultivation of TE 671 muscle sarcoma cells. Dulbecco's modified Eagle's medium (4.5 g/l glucose) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin, and streptomycin was used. A commercially available bubble jet print head was used to apply single or multiple droplets of ~25 pl volume to the bottom face of the membrane (Fig. 1) (6).

Mixtures of calcein-AM (0.25 mM, nonfluorescent) and eosin Y dye (0.1 mM) and of acetylcholine (1 mM) and eosin Y dye (10 μM) were used. Eosin Y served to indicate the distribution and the diffusive transport of the active, yet invisible substance (calcein-AM or acetylcholine, respectively) in the medium. Cellular response was recorded using a Zeiss Axiophot fluorescence microscope (Zeiss, Göttingen, Germany) fitted with a Hamamatsu C2400-08 video camera (Hamamatsu Photonics, Herrsching, Germany) employing 485 nm excitation and 515 - 585 nm emission filters. Calcein AM becomes fluorescent after enzymatic hydrolysis of ester residues after its incorporation into cells. To monitor excitation by acetylcholine, cells were first loaded with Ca^{2+}-sensitive fluo-4-AM dye (Invitrogen, Karlsruhe, Germany) by incubation for 30 min at a concentration of 2.3 μM. Subsequently the medium was changed to remove extracellular fluo-4-AM dye and cells were incubated for another hour at 37°C. Upon excitation, intracellular Ca^{2+} concentration increases resulting in an increase of fluorescence intensity. The latter is determined using Image J software in regions of interest of 10 μm diameter manually specified for each cell (cf. Fig. 3, $t = 5$ s).

RESULTS

Application of calcein-AM/eosin Y: TE 671 cells were cultivated on nanoporous membranes (pore size, 1 μm) and stained with Hoechst 33342 dye (nucleus staining) to track cell location in the membrane (Fig. 2 *A*). The calcein-AM/eosin Y mixture (5 droplets, 125 pl total volume) was applied resulting in a transient fluorescence signal indicating the spot size ($\approx$60 μm) where substance was actually delivered to the cell layer. Control experiments with eosin Y did not show any uptake of this dye into TE 671 cells. Rather, eosin Y fluorescence vanishes over a period of several minutes to invisibility due to diffusion in the medium (Fig. 2 *D*). After this time period, only cells appear fluorescent, which is attributed to the incorporation and enzymatic hydrolysis of calcein-AM by the cells (Fig. 2 *D*). At the concentration employed here (0.25 mM), only cells located directly above the application spot were sufficiently loaded by calcein to show appreciable

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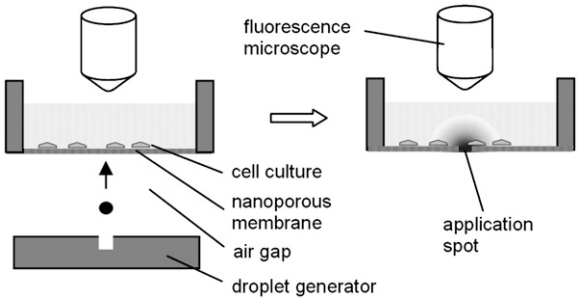


FIGURE 1 Experimental setup for localized chemical stimulation of cells that grow on a nanoporous membrane forming the bottom of a tissue chamber. Either a single or a series of microdroplets may be generated and shot at the bottom face of this membrane by a bubble jet device at <10 μs temporal resolution. The location of the application spot (diameter typically ≈ 60 μm at 25 pl droplet volume) is adjusted by an *xy*-stage. The fluid penetrates the nanopores and either directly stimulates a cell growing on these pores or diffuses in the medium to cells in the periphery and eventually elicits cellular responses that are monitored by fluorescence microscopy.

fluorescence. Variation of concentration and droplet volume thus determines effective spatial resolution achievable by this chemical stimulation scheme.

Acetylcholine stimulation: single droplets of a mixture of acetylcholine (1 mM) and eosin Y (10 μM) were applied to the bottom of a nanoporous membrane (pore size, 50 nm). Fig. 3 shows a panel of fluorescence micrographs of the cell culture after the application of the droplet (at $t = 0$ s). The eosin Y fluorescence indicates the location of the application spot (*center*). Subsequently, fluorescence of TE 671 cells appears and shows a wave of excitation progressing from the

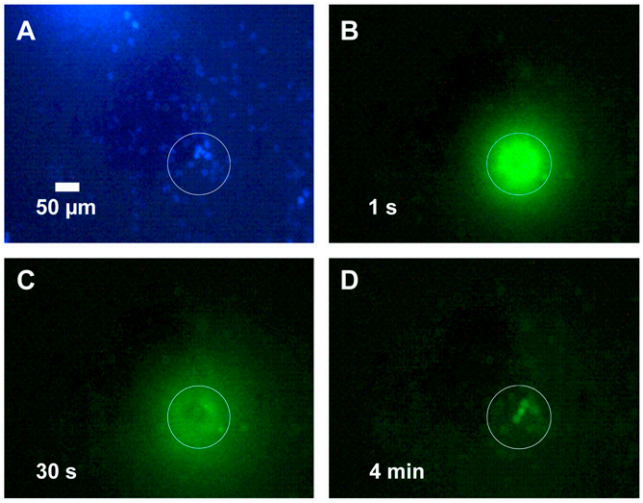
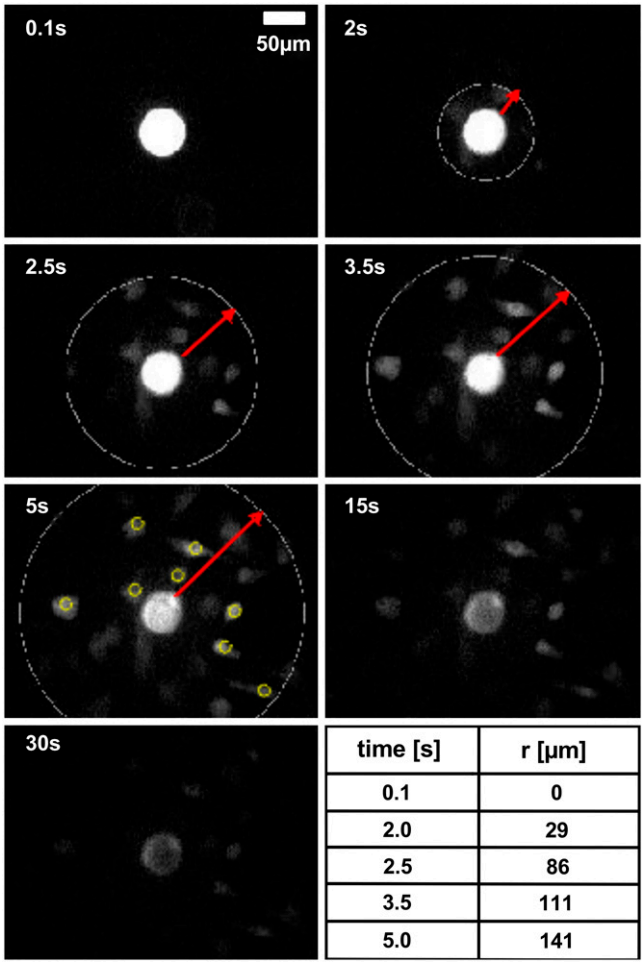


FIGURE 2 Chemical stimulation by calcein-AM / eosin Y. (*A*) Micrograph of cell culture before stimulation in Hoechst 33342 fluorescence. (*B*) Eosin Y fluorescence 1 s after deposition of droplets. (*C*) Eosin Y diffuses in the medium and fluorescence intensity decreases. (*D*) After 4 min, intracellular calcein fluorescence is retained, whereas eosin Y fluorescence has decayed.



time [s]	r [µm]
0.1	0
2.0	29
2.5	86
3.5	111
5.0	141

FIGURE 3 Panel of micrographs showing the application of a single droplet of acetylcholine (1 mM)/eosin Y (10 μM), the dilution of eosin Y as a result of diffusion, and the evolution of a circular excitation pattern in the cell layer visualized by Ca^{2+} imaging. The small circles in the graph at $t = 5$ s indicate the location of cells whose fluorescence intensity is plotted as a function of time in Fig. 4. Additional data is provided in Table 1.

application spot to the periphery in a circular symmetry. In the figure inset, the effective radius of excitation is given as indicated by arrows in the micrographs. After 30 s, fluorescence has almost completely decayed. Fluorescence intensity traces of the cells indicated by circles in Fig. 3 ($t = 5$ s) were analyzed in detail (Fig. 4, Table 1). The delay, τ, of cellular response with respect to droplet delivery varies proportional to the square of the distance, d, between the rim of the application spot and the location of the cell under investigation as is expected for diffusive transport (Fig. 4 *inset*, Table 1).

The apparent lag time of 1.5 s between stimulation and onset of excitation of cells (*inset*, Fig. 4) is attributed to intracellular signaling in agreement with earlier reports (7). The low level of fluorescence intensity at $t < 1.5$ s confirms that contribution of eosin Y to overall fluorescence is negligible outside the application spot. Interestingly, cells showed different maximum intensities, even if located at similar distances d. This

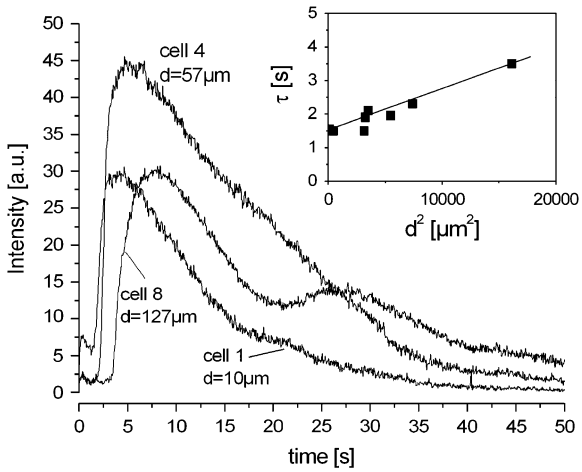


FIGURE 4 Temporal evolution of the fluorescence intensity (fluo-4-AM dye) in cells as indicated in Fig. 3 (traces of only three cells plotted). The inset shows the delay, τ, in the response of cells as a function of the distance, *d*, between the rim of the application spot and the cell for all cells indicated in Fig. 3.

may be due to different degrees of loading with fluo-4-AM dye. The origin of the modulation in some of the fluorescence signals (for example, cell 5, Fig. 4) is yet unknown.

The response time as it may be determined from the initial slope (from 10% to 50% of the final fluorescence signal intensity seems to show a tendency toward an increase with increasing d). This would point to a dose/response relationship as acetylcholine concentration is expected to decrease with increasing d. However, further study in cell cultures with a larger field of view will be necessary to confirm this preliminary finding. At lower concentration of acetylcholine, excitation is limited to a smaller area or even precisely to the application spot (data not shown). This finding is as expected, since half spherical diffusive transport of acetylcholine in the space above the cell layer will result in progressive dilution of the neurotransmitter. At a certain distance from the application spot, the concentration will be lower than the threshold level required to induce excitation of cells. Also, the use of smaller droplets would further enhance spatial resolution.

CONCLUSIONS

Functional chemical stimulation of adherent cells may be achieved in a well-controlled and versatile fashion by utilizing nanoporous membranes as support and localized substance application by bubble jet technology. By variation of concentration and/or volume of the fluid, spatial resolution and range of excitation may be controlled. Future research will be directed toward numerical simulation of concentration profiles along the surface of the cell layer considering the application spot as extended drainable reservoir and the measurement of complete dose/response curves from experiments as demonstrated in this Letter. As the cellular response reflects the local concentration, this approach should allow for rapid determination of dose/response curves in a single experiment as opposed to the multi-well plate approach commonly used today.

TABLE 1 Kinetic of excitation

Cell no.	Distance from application spot d (μm)	Delay τ (s)	Maximum intensity (a.u.)
1	10	1.55	30.5
2	21	1.5	24.1
3	56	1.5	43.6
4	57	1.9	44.3
5	59	2.1	44.6
6	74	1.95	26.5
7	86	2.3	43.7
8	127	3.5	29.1

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