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Functional Screening of Intracellular Proteins in Single Cells and in Patterned Cell Arrays Using Electroporation

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A tool for detection and characterization of intracellular enzyme−substrate and receptor−ligand interactions inside the cytoplasm of single targeted cells or small confined groups of cells is presented. Fluorogenic enzyme substrates and receptor ligands were rapidly delivered by electroosmosis and internalized by electroporation in cells using an electrolyte-filled capillary (EFC) biased at a high voltage. Specifically, alkaline phosphatase and proteases were detected in single NG108-15 cells using fluorescein diphosphate and casein BODIPY FL, respectively. The intracellular 1,4,5-inositol triphosphate (IP_3) and ryanodine receptors were detected after EFC introduction of the selective receptor agonists IP_3 and cyclic adenosine diphosphate ribose (cADPr), respectively. Receptor activation in both cases resulted in increased cytosolic concentrations of free calcium ions that were measured using the calcium-ion-selective probe, fluo-3. The effect of cADPr could be blocked by coadministration of the ryanodine receptor antagonist ruthenium red. Furthermore, electroporation of a plurality of cells grown in microwell structures ($100 \times 100 \times 45$ μm) molded in PDMS is demonstrated. The methods and systems described using an EFC for electroporation and delivery of protein markers, ligands, and substrates might be useful in high-throughput screening of intracellular targets, with applications in proteomics and phenotype profiling, as well as in drug discovery.

Proteins constitute ∼18% of the total weight in mammalian cells and are of central importance in supporting cell function and structure. Because of the tight regulation between different enzyme systems, and signaling pathways, a protein is often capable of imposing an effect on a large number of different reactions and interactions within a single cell. Sometimes these interactions between different signaling pathways result in nonintuitive dynamical behaviors, such as oscillations in product formation.[1] Several factors, such as age, chemical and physical signals, and environmental cues, affect protein expression and function. This biological variation in expression and processing of intracellular proteins spans over vastly different time scales, from milliseconds to years, and involves a complex palette of different chemical and physical reactions and interactions. Methods for detecting protein expression and function are central in characterizing and understanding many aspects of cell biology and for drug discovery. In particular, rapid, cell-based, and functional screening methods that allow characterization of interactions between intracellularly confined proteins and small molecules would dramatically increase the target space for drug libraries. Today drugs are usually tested for targets situated on the cell plasma membrane, such as receptors, ion channels, and transporters. These, however, represent a minority of the proteome, the rest residing inside the cell. A powerful analytical tool for identification of large numbers of proteins is based on separation with polyacrylamide gel electrophoresis (2D PAGE) followed by detection by MALDI-TOF MS.[2] This technique has the potential for full-proteome global mapping in many organisms. Expression of cytosolic and organelle-bound proteins and peptides in single cells can additionally be detected and interrogated using miniaturized chemical analysis in combination with ultrasensitive detection protocols. For example, intracellular proteins, peptides, and catecholamines in the cytoplasm of single cells have been detected using capillary electrophoresis coupled to laser-induced fluorescence[3] and electrochemical detection[4] and through MALDI-TOF MS mapping.[5] Detection of proteins, peptides, and other small molecules have been performed on the single-organelle level using MALDI-TOF MS,[6] CE coupled to optical detection,[7] and electrochemical detection of individual secretory vesicles.[8] Also, PCR has been performed on single cells for the investigation of cell-to-cell variations in a population of cells.[9]

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All said detection schemes and microchemical methods are helpful in determining the protein content of cells but yield very little functional information. Ideally, protein expression and function should be studied inside living cells. Highly specific fluorogenic enzyme substrates and protein probes including reporter proteins, such as green fluorescent protein (GFP),[10] luciferases, and β-galactosidases, make it possible to detect proteins and investigate, for example, protein−protein interactions in complex biological samples, including living cells. There are a variety of substrates available that can be employed as light switches in the substrate−product conversion step. Examples include probes for detection of proteases, phosphatases, peroxidases, sulfatases, peptidases, and glycosidases. Either the substrate is fluorescent and the product nonfluorescent or vice versa. Also, specific protein−protein interactions can be identified by the use of a fluorescence indicator coupled to protein splicing.[11] In addition, interactions of nuclear membrane proteins with chromosomes have been investigated by four-dimensional imaging[12] and by the use of fluorescent-protein biosensors.[13] Furthermore, an extracellular stimuli acting on surface targets (receptors, enzymes, transporters, ion channels) may trigger a cascade of events in the cell, including change in the intracellular free calcium ion concentration, which can be used in fluorescence detection of a variety of compounds evoking such responses.[14−17]

Enzyme systems coupled to intrinsic fluorescent compounds such as the cofactors NADH, NADPH, and flavins or amino acids such as tryptophan can also be used for monitoring enzyme activity down to the level of single enzymes.[18]

Since most proteins are contained within cells and most protein labels, drugs, and transfection agents are membrane-impermeant, techniques such as electroporation, lipofection, and microinjection[19] have been used for their entry into cells. Electroporation uses a transient electrical field that causes an increase in membrane permeability by formation of pores. Electroporation is usually performed in bulk solution, but techniques that allow the manipulation of single cells have been developed recently.[20−23]

We herein propose a new concept for rapid functional screening and probing of intracellular proteins in living cells. Fluorogenic substrates of enzyme activity and receptor ligands (agonists and antagonists) for identification of intracellular receptors by indirect activation of fluorogenic probes are delivered to the cell surface and introduced into the cytosol of a living cell using a recently developed single-cell electroporation technique that utilizes an electrolyte-filled capillary (EFC) made of fused silica.[20] The cellular membrane is permeabilized only during the voltage pulse, avoiding excessive leakage of cytosolic components. Enzyme and receptor activity is monitored using fluorescence microscopy. The method enables screening of intracellular protein content and function as efficiently as methods deviced for cell-surface targets and can be multiplexed to a highly parallel format.

EXPERIMENTAL SECTION

Chemicals. Fluo-3 AM ester, casein BODIPY FL, and fluorescein diphosphate (FDP) were purchased from Molecular Probes (Leiden, The Netherlands). Cyclic adenosine diphosphate ribose (cADPr), ruthenium red (RR), 1,4,5-inositol triphosphate (IP_3), and the chemicals used for buffer solutions were all of analytical grade and purchased from Sigma (St. Louis, MO). Materials for poly(dimethylsiloxane) (PDMS) membrane fabrication were Nano SU-8 25 (MicroChem Corp, Newton, MA), XP SU-8 developer (Microresist Technology GmbH, Berlin, Germany), and Sylgard 184 (Dow Corning, Midland, MI). Methanol pro analysi, and ethanol 95% dimethyldichlorosilane and trichlorethylene, pro analysi, were purchased from Sigma.

Cell Culturing and Preparation. NG108-15 cells were grown as previously described.[24] Before experiments, they were plated on a no. 1 cover slip and allowed to grow for 1−3 days. Cell dishes were mounted in a circular polycarbonate holder and transferred to the stage of a microscope. Prior to experiments, the culture medium was replaced by a HEPES buffer (NaCl 135 mM, KCl 5.0 mM, $MgCl_2$ 2.0 mM, $CaCl_2$ 2.0 mM, D-glucose 10 mM, HEPES 10 mM; pH was adjusted to 7.4 with NaOH). All buffer solutions were made in water from a Milli-Q system (Millipore).

For the cADPr and IP_3 experiments, fluo-3 AM ester staining was performed by incubating the cells for 30 min in dye solution (10 μM fluo-3 AM ester in HEPES buffer) at room temperature. To remove excess uncaptured dye, the cells were washed three times in HEPES buffer and stored for an additional 30 min in HEPES buffer before the experiment.

Electroporation. Electroporation was performed as previously described[20] with minor modifications. The experimental setup is schematically depicted in Figure 1. Briefly, the electrolyte-filled capillary (30 cm long, 30−50 μm i.d., 375 μm o.d.) was positioned 20−40 μm above the cell using a high-graduation micromanipulator (Narishige, MWH-3, Tokyo, Japan). To position the EFC at a specific distance from the cell surface, the cell was first brought in focus by observing the cell in the microscope. Then the focus was changed using the micrometer markers on the focusing knob of the microscope to the desired distance above the cell. The EFC was lowered until the lumen of the EFC came in focus. After positioning of the EFC tip, the focus was returned to the cell. The cell-bathing medium was grounded with a platinum wire. A pulse was applied with a dc high-voltage power supply (model ARB 30, Bertan, Hicksville, NY) for a duration of 5−60 s.

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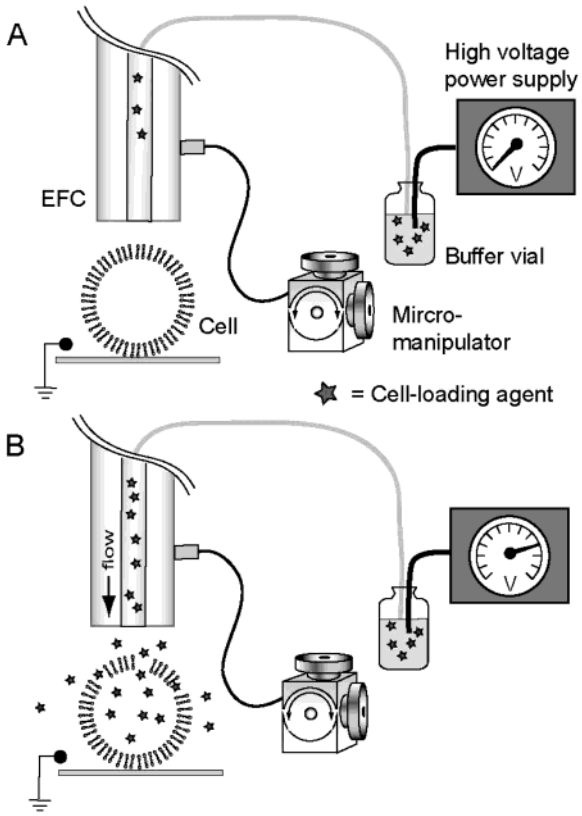

Figure 1. (A) Schematic picture of the experimental setup for introduction of protein markers into cultured cells. Cover slips with cultured cells were mounted in a chamber on the microscope stage. The outlet end of an electrolyte-filled capillary (EFC) was positioned above the cell surface (20−40 μm) using a micromanipulator. The inlet end of the EFC (anode) was placed in a buffer vial, and a platinum wire was placed in the cell bath solution, serving as a ground electrode. (B) Dc potential pulses were applied by a high-voltage power supply. The residual electric field at the EFC outlet results in the formation of transient pores in the cell plasma membrane. At the same time, membrane-impermeant cell-loading agents supplemented to the electrolyte solution contained in the EFC, migrate out from the EFC with the induced electroosmotic flow and enter the cell interior by diffusion through the membrane pores.

The EFC was filled with the desired solution, i.e., cADPr (500 μM), RR (1 mM), IP_3 (100 μM), FDP (500 μM) or casein BODIPY FL (100 μg/mL), and an intracellular buffer (NaCl 5 mM, KCl 135 mM, HEPES 20 mM, $MgCl_2$ 1.5 mM, D-glucose 10 mM; pH was adjusted to 7.4 with NaOH) was used as electrolyte. In control experiments, the EFC was filled with intracellular buffer solution, which was electroporated into the cells. In all cases, except for IP_3, a 50-μm-i.d., 30-cm-long EFC was used and 10 kV was applied over the EFC. For IP_3, a 30-μm-i.d. EFC was used and 10 kV applied. The substrate FDP contained traces of fluorescein monophosphate, causing background fluorescence. Therefore, cells were bleached during the electroporation pulse (pulse length 5 s) plus 10 s after the electroporation event to eliminate the background. The cells were viewed 30 s after electroporation and thereafter periodically in 30-s intervals. Casein BODIPY FL was electroporated with a 10-s pulse and viewed at intervals of 30 s after the electroporation event. In FDP and casein BODIPY FL experiments, the EFC was moved from the cell before viewing. For cADPr measurements, a voltage pulse length of 20 s was used. IP_3 was introduced to cells by applying a 10-kV pulse for 1 min. In the screening for the effects of IP_3, cADPr, and RR, a background was first measured for 30−60 s. The pulse was applied and the event after followed until the baseline returned to the original level. For electroporation in wells, the EFC was positioned 40 μm over the cells and a 12-kV pulse with 10-s duration was applied. Directly after the electroporation event, the wells were washed with HEPES buffer to get rid of impurities of fluorescein monophosphate and the EFC moved. The cells were viewed 1.5 min after the pulse had been applied. Control runs were made by electroporating an empty well with FDP and performing the washing procedure after the electroporation event.

Cellular Array Membrane Fabrication. The membrane pattern was drawn in AutoCAD format and transferred to a chrome mask using e-beam (JBX-5DII, JEOL Ltd., Tokyo, Japan) lithography and wet etching in a cleanroom facility.

Polished ⟨100⟩, oriented, unoxidized 3-in. silicon wafers were used as masters for molding. The membrane pattern was transferred to an ultrathick negative photoresist (SU-8 25), thus forming a positive relief mold, using a standard UV lithography technique.[25] The resist was developed in XP SU-8. The desired membrane thickness is ∼45 μm, and the actual thickness of the SU-8 structures was examined with a surface profilometer (Tencor Alpha Step 500, KLA-Tencor, San Jose, CA). For mold release, the master was silanized in a 1:10 mixture of dichlorodimethylsilane and trichloroethylene.

Thin membranes of PDMS, carrying an array of wells (Figure 4A), were fabricated with the sandwich molding principle.[26] A 10:1 mixture of PDMS oligomer and curing agent (Sylgard 184) was degassed under vacuum and a couple of drops of PDMS were placed onto the molding master that was to be replicated. Silanized microscopy slides were placed slowly on top of the drops, allowing surface tension to pull the slide down, avoiding the formation of bubbles. The PDMS was slowly pressed out over the area. Lead pieces were placed on top of the assembled package of supporting glass plate, master, PDMS, and glass slides. The total package was placed in the curing oven at 80 °C for 1 h. After curing, the pressure was removed and the microscopy slides were removed. The resulting membrane was peeled way from the master and stored for further treatment.

Instrumentation. Excitation of products formed in experiments with FDP (both single cells and wells) and casein BODIPY FL was performed with an Ar^+ laser (Spectra-Physics 2025−05, Sunnyvale, CA). The laser beam was sent through a spinning disk to break the coherence, a 488-nm line interference filter (Leica I-3 filter cube), and focused onto the cover slips using a 40× objective (Leica 0.9 NA) mounted in an inverted microscope (Leica DMIRB, Wetzlar, Germany). Images were recorded with a three-chip color CCD-camera (Hamamatsu C6157, Kista, Sweden).

Intensity measurements in experiments with cADPr, RR, and IP_3 were performed with a SPEX CM-X spectrophotofluorometer (SPEX Industries, Inc., NJ) equipped with a 150-W Xe lamp for excitation (488 nm) and connected to an inverted microscope (Leica DMIRB). The fluorescence emission (Leica I-3 filter cube) was imaged onto a photomultiplier tube (PMT) with a photomi-

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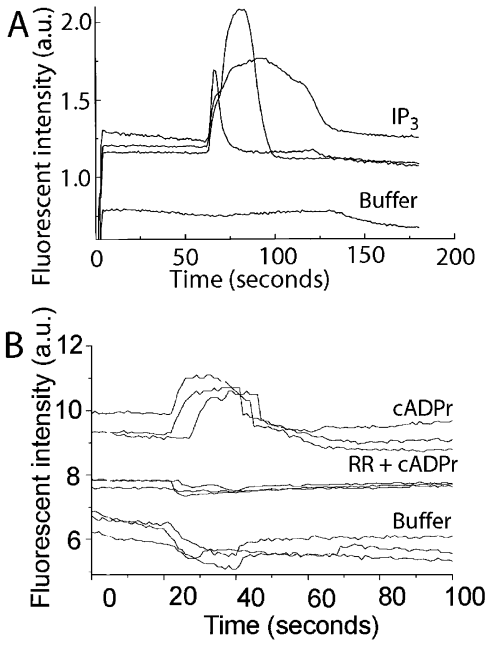

Figure 2. Identification of intracellular receptors in fluo-3 AM (10 μM)-stained NG108-15 cells. (A) Detection of the IP_3 receptor by EFC introduction of the agonist, IP_3 (100 μM, pulse length 60 s, HV = 10 kV). The response curve had different shapes, and three examples are shown here. The lower trace is a blank run. The pulse was applied after 60 s and a 30-μm-i.d., 30-cm-long EFC was used. The response rate was 100% (n = 6). (B) Upper trace shows identification of the ryanodine receptor by adding cADPr (500 μM) to the electrolyte of a 50-μm-i.d., 30-cm-long EFC; 10 kV was applied for 10 s. Upon activation, the ryanodine receptor triggered a release of calcium from ER, which binds to fluo-3, and the resulting increase in fluorescence was measured. Different cells responded differently to the stimulation and three response curves are shown. The response rate was 60% (n = 17). The voltage was applied after 20 s. In the middle traces, cADPr and RR (1 mM) were coelectroporated into the cell. No response was obtained since RR is blocking the calcium channel. The success rate was 100%, n = 6. Lower traces are blank runs where intracellular buffer was introduced using the EFC. A small decrease in fluorescence can be observed due to leakage of dye through the formed pores.

crographic attachment (Nikon AFX-DX, Bergström Instruments, Stockholm, Sweden).

RESULTS AND DISCUSSION

Detection of Intracellular Ligands and Receptors. The agonist IP_3 was used to screen for intracellular IP_3 receptors inside intact living cells. IP_3 (100 μM) was added to the calcium-free electrolyte of an EFC. When the high-voltage pulse was applied over the EFC, pores were formed in the cell plasma membrane while the introduction of the agonist into the cell was facilitated by concentration gradients and the electroosmotic delivery of the agonist toward the cell surface.[20] When the agonists bind to the IP_3 receptors on the endoplasmic reticulum (ER), calcium is released into the cytosol as seen in the upper fluorescence trace in Figure 2A. The initial part of the curve is steep, corresponding to the fast activation of the calcium ion-permeable channels on ER, dramatically increasing the free cytosolic calcium concentration, which thereafter is pumped back into the ER by Ca^{2+}-ATPases. The measured dissociation constant (K_D) for IP_3 acting on the IP_3 receptor/channel ranges from <1 to ~100 nM[27−30] and EC_{50} values reported for IP_3 are in the range of 80−260 nM.[31]

In the same way, the ryanodine receptor agonist cADPr (EC_{50} = 18−48 nM,[32,33] K_D = 176 nM[34]) was introduced to NG108-15 cells. Cytoplasmic administration of cADPr evoked a release of calcium through the ryanodine receptor,[35] which is seen in the upper trace in Figure 2B. To verify the specific receptor activation, selective blocking of the receptors can be performed. This is here shown by coelectroporating RR (1 mM), together with cADPr (500 μM). RR is a high-affinity antagonist of ryanodine receptors[36,37] and blocks these channels by lodging in the pore[38] with an IC_{50} value of 6.85 nM.[39] As seen in the middle trace in Figure 2B, the RR concentrations used efficiently blocks the action of cADPr on ryanodine receptors and there is no calcium release observable in the fluorescence trace. The blank run shows a small dip in the baseline when the voltage pulse was applied (see the lower trace in Figure 2B). This is probably due to leakage of the dye during the permeabilization of the membrane. Using dyes such as mag fura-2, which enters the ER, such effects are likely to be avoided or minimized. Selective receptor blockers or antagonists are also extremely useful for performing competitive assays. For example, 8-NH2-cADPR is a competitive antagonist for the ryanodine receptor with an IC_{50} of 10 nM.[40] By coelectroporating agonists together with inhibitors or blockers, titration of the response can be performed within the cell in order to investigate the pharmacology of the intracellular receptor systems, i.e., to determine whether a drug is a competitive or noncompetitive drug and to obtain kinetic information as well as IC_{50}, and EC_{50} values.

Except for identification of intracellular receptors as demonstrated above, so-called ligand fishing or deorphaning can be performed in a similar way. A cell with a known set of receptors is used as a detector cell, and a library of ligands is introduced to the cell. Also, identification of markers for diagnostics and monitoring of disease progression as well as identification of therapeutic agents are judged to be feasible with this technology.

Enzyme Identification in NG108-15 Cells. Single NG108-15 cells were electropermebilized for the introduction of fluorogenic substrates that target specific enzymes or groups of enzymes. Specifically, FDP (500 μM) was used to target the intracellular enzyme alkaline phosphatase that catalytically hydrolyses the phosphoester bonds on the substrate so that the highly fluorescent product fluorescein is formed in the cytosol (Figure 3A−C). Also, the intracellular protease activity was

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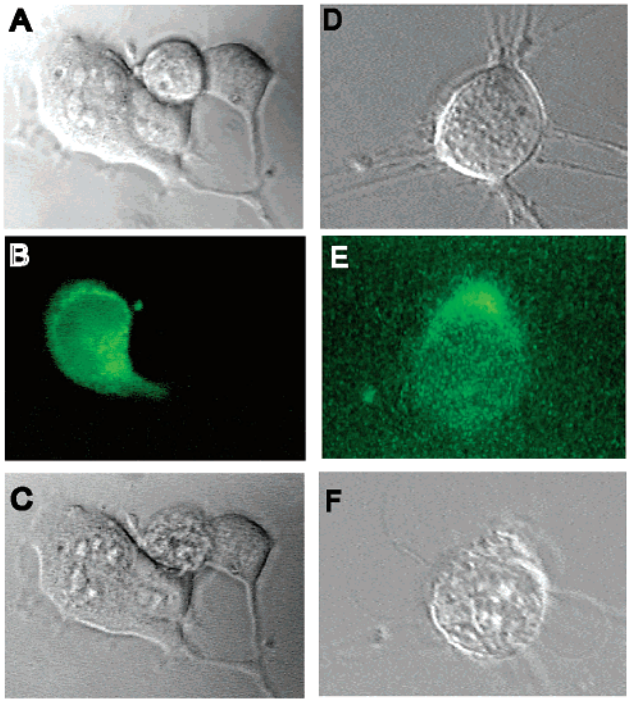

Figure 3. Detection of alkaline phosphate in NG108-15 cells. (A) A cell was chosen and the substrate fluorescein diphosphate (FDP) was supplemented to the electrolyte of the EFC. (B) A high-voltage pulse (5 s, 10 kV) was applied. 30 s after the electroporation event; the fluorescence was measured with a charged coupled device (CCD) camera. The response rate was 70% ($n = 8$). (C) The same cell after the electroporation event. Images D−F show the detection of proteases in a single NG108-15 cell. Casein BODIPYFL was introduced (10 s, 10 kV) and fluorescence was monitored 30 s after electroporation. Here, the response rate was 60%, $n = 19$.

investigated using a protein, casein (100 μg/mL), heavily loaded with the green fluorescent molecule BODIPY FL, as substrate. In solution, casein BODIPY FL is folded so that the quartenary arrangements in the molecule quench the fluorescence. When the peptide bonds are cleaved by the action of cytosolic proteases, segments of highly fluorescent peptide adducts tagged with BODIPY FL are released, Figure 3D−F. In both cases, enzyme activity can be correlated to the increase in fluorescence, which means that this method is suitable for screening and determination of enzyme activity in single cells. In particular, protocols such as these can be used in single-cell proteomics where differences in enzymatic activity would, for example, be useful in revealing phenotypes in a cell population.

Similar to employment of antagonists in the receptor screening described above, selective enzyme inhibitors can be used to further characterize enzyme function and to increase the specificity in an intracellular enzyme assay. Also, strategic blocking of particular enzymes can efficiently block entire signaling pathways.[15] The use of specific inhibitors or modulators might enable control of cellular processes and provide leads for identifying novel enzyme systems.[16]

Enzyme Identification in Cells Grown in Microwells. To employ EFCs in a multiplexed and parallel screening format it is important to be able to address and manipulate small populations of single cells in a controlled way. Preferably, distinct groups of cells cultured in microwells are used for this purpose. Such arrays

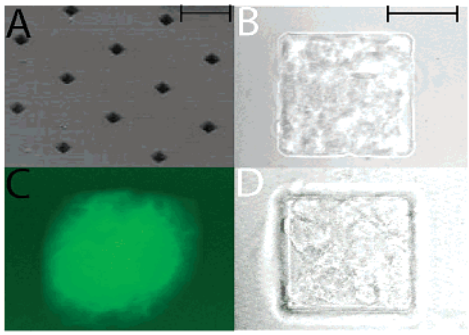

Figure 4. Electroporation of a confined cell population (A) in a PDMS membrane with microwells, size 100 × 100 × 45 μm. The distance between the wells is 200 μm. Scale bar, 200 μm. (B) Bright-field image of cells in a microwell. Scale bar, 50 μm. (C) A pulse (12 kV, 10 s) was applied and FDP (500 μM) was electroporated into the cells in the well. (D) Shows the same well after electroporation. The response rate was in this case 100%, $n = 6$ and control runs were negative.

of confined populations of surface-immobilized cells can be manufactured by microcontact printing of cell-anchoring proteins[41] by the use of microwells.[42] Here NG108-15 cells were cultivated in PDMS microwells, and Figure 4 shows how FDP is introduced using EFC electroporation into several cells present in a microwell. The dimension of a well is 100 × 100 × 45 μm, and it houses a monalyer of ∼50 cells, which is sufficient for obtaining statistically relevant information of the entire cell population. The wells can easily be made in different sizes even to fit a single cell.[43] To perform high-throughput screening of intracellular protein targets it is necessary to perform parallel analyses. For this purpose, standard 96-well plates can be used in combination with matching 96-capillary arrays,[44] for addressing single or a defined number of cells within each well.

CONCLUSION

A tool for profiling, screening, and probing of intracellular proteins has been developed. It is demonstrated that intracellular systems in living cells are easily accessed using an EFC for the delivery, introduction, and confinement of otherwise membrane-impermeable indicators of protein activity and protein activators (receptor ligands and enzyme substrates). In this way, individual proteins and biological pathways can be interrogated and studied in their natural habitat.

It was also shown that, using soft lithography, an array of wells for housing small populations of cells could be produced in PDMS. In combination with a single EFC for fast sequential delivery of cell-affecting agents or a plurality of EFCs for parallel delivery of cell-affecting agents, screening and drug targeting of intracellular receptor and enzyme systems as well as investigating cell-to-cell variations in cell populations can be performed rapidly. Multiplexed EFC electroporation of a plurality of cells grown in arrays might become a useful tool in the search for agents such as drugs,

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genes, antisense oligonucleotides, etc., that affect intracellular chemistry and for the detection of pathophysiological states using specific markers.

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