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Short communication

Development of a scanning electrochemical microscopy-based micropipette and its application to analysis of topographic change of single-cell

Yu Hirano, Keiko Kowata, Mikie Kodama, Yasuo Komatsu *

Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira, Sapporo, Japan

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ABSTRACT

Scanning electrochemical microscopy (SECM) is useful for analyzing various cellular responses. We have combined a micropipette (MP) with SECM to perform quantitative solution delivery to single cells. In this system, since the concentrations of electrochemical mediators are changed by the volume of solution delivered from the MP, we constructed a feedback control system to regulate MP delivery by SECM-detected signals. Cellular responses induced by MP delivery could be monitored by the SECM, and cell apoptosis was successfully detected by adding a kinase inhibitor of two orders of magnitude less than what is required in the conventional method. The SECM-based MP can activate a target cell, requiring a minimal amount of agent, and can continually examine target cell responses. This system improves the accuracy of delivery from the MP and is useful for single-cell analysis.

1. Introduction

Living cells exposed to biologically active molecules frequently undergo physiological and morphological changes. Shrinkage and swelling are major cellular responses observed in early phase apoptosis [1–3] or necrosis [4]. These changes can be induced by specific exogenous molecules depending on the concentration and exposure duration. Usually, active molecules have to be dissolved in the entire culture medium, even for single-cell analysis. Micropipettes (MP) have been developed to reduce the quantity of active agents consumed, and most use the pressure ejection method [5,6], which employs a positive pressure pulse to eject a small volume of solution. However, the concentration of delivered agent, to which the cell is exposed, is altered because the MP tip is moved slightly by the pressure change. The temperature expansion of a liquid alloy [7], or electroosmosis [8], has been developed to control MP delivery. The microjet electrode has also been developed to analyze the mass-transport rates from MP [9], but the concentration of the biologically active agents around the target cells has not been controlled.

Microelectrodes have been used to evaluate chemical events at the cell surface [10–12]. Scanning electrochemical microscopy (SECM) allows the electrochemical profiling of a local area via microelectrode scanning [13–15]. It has been applied to detect membrane permeability [16–18], cellular topography [6,19], and intra- and extra-cellular reactions essential for cell survival [20–23]. We have developed a novel MP system to control the concentration of biologically active agents around target cells using SECM. The SECM-detected current reflects the concentration of the electrochemical mediator, and the concentration is influenced by the solution volume delivered from the MP. We constructed a feedback control system to regulate MP delivery using the SECM-detected current. Following solution delivery, SECM was used to monitor the response of the target cell. Apoptotic cell shrinkage was observed to be dependent on the staurosporine (STS) concentration, which induces apoptosis via the inhibition of a broad spectrum of kinases [3,24,25].

The SECM-based MP (SECM-MP) was constructed to examine the responses of a single cell with low amounts of biologically active agents. This system can be suitable for various cellular analyses because it provides reliable and reproducible results.

2. Material and methods

2.1. Cell lines and materials

HepG2 (human hepatoma) cells were grown as a monolayer culture in Dulbecco's modified Eagle's medium (Sigma, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) in flasks at 37 °C in a humid atmosphere containing 5% CO_2 in air. Cultivated cells grown in a 35 mm dish (Nippon Genetics, Japan) at a density of 1×10^4 to 3×10^4 cells/well were cultured overnight. Before the experiment, the cells were washed with HEPES buffered saline (HBS; 150 mM NaCl, 4.2 mM KCl, 2.7 mM $MgCl_2$, 1 mM Na_2HPO_4, 11.2 mM glucose, and 10 mM HEPES; pH 7.2) and incubated them in the measurement solution (1 mL). Calcein acetoxymethyl ester (calcein-AM; Dojindo, Japan), hydrogen peroxide (Wako, Japan), and staurosporine

* Corresponding author. Tel.: +81 11 857 8437; fax: +81 11 857 8954.
E-mail address: komatsu-yasuo@aist.go.jp (Y. Komatsu).

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(STS; Sigma) were used for delivery experiments. All other reagents were of analytical grade, and solutions were prepared with ultrapure water from a Millipore system.

2.2. Instrumentation

SECM measurements were performed with an instrument built in our lab, as described previously [19]. Briefly, this SECM setup consisted of a piezo-motor positioning system (Physik Instrumente, Germany), an inverted microscope, a temperature-controlled bath, PXI system for data acquisition and instrument control (National Instruments (NI), TX, USA), and a potentiostat (Hokuto Denko, Japan). To control the delivery rate from the MP, the microinjector (Narishige, Japan) injection pressure was mechanically controlled by a stepping motor and a motor controller (Suruga Seiki, Japan). The MP tip position was controlled by an XZ stage at a scan rate of 100 μm/s (Suruga Seiki). The SECM-MP system software was constructed with LabView (NI). MPs were fabricated from borosilicate capillaries (o.d./i.d. = 1.0/0.6 mm with an inner filament; Narishige, Japan) using a micropipette puller (Narishige). MPs with opening diameters between 10 and 12 μm were fabricated using a microforge (Narishige), and the exact opening sizes were measured using a digital microscope (Keyence, Japan). We fabricated a Pt microdisk electrode (5 μm radius) by heat sealing the Pt wire in a borosilicate glass capillary. The radius of the Pt electrode, including the glass sheath, was 10 μm (RG = 2). We used an Ag/AgCl (3 M KCl) electrode as the reference and auxiliary electrode. We applied a 0.5 V (vs. Ag/AgCl) potential to the probe electrode for the oxidation of $K_4[Fe(CN)_6]$, and the temperature was set at 34 °C for measurements of living cells.

2.3. Control of MP delivery

The MP delivered a solution, which did not contain any electron mediators from upstream of the microelectrode, into HBS containing 4 mM $K_4[Fe(CN)_6]$. In this condition, $K_4[Fe(CN)_6]$ oxidation currents decreased depending on the delivery rate. We constructed a feedback control system between the microelectrode current signals and the microinjector injection pressure to control the MP delivery (Fig. 1A). We used the microelectrode with the same tip RG (RG = 2) for feedback control. The microelectrode tip at the downstream position of MP (Fig. 1B) was not moved during the control. We measured current responses during MP delivery with and without feedback control to evaluate the SECM-MP system. When the MP delivered without feedback control, the injection pressure was maintained constant, whereas when it delivered with feedback control, the feedback current was set at 50% of the initial current. The current ratio ($I_r = I \cdot 100\%/I_{t0}$), where I_{t0} is the initial current and I is the current at the time of measurement, was used for analysis. We also measured current responses during MP delivery at various delivery rates with it and interruption repeated at 3-min intervals, and feedback currents were set at 40%, 50%, 60%, 70%, 80%, and 90% of the initial current. To stimulate target cells via MP delivery, the MP and microelectrode were automatically positioned as shown in Fig. 1B. After delivery, the MP tip was arranged at a vertical distance of 1100 μm from the substrate surface (Fig. S-1).

2.4. Staining living cells via MP delivery

Calcein-AM was used to stain living cells via MP delivery. It passively crosses the cell membrane in an electrically neutral form and is converted by an intracellular esterase into a negatively charged form—fluorescent calcein—which is retained in the intracellular compartment as long as the plasma membrane remains intact [26,27]. We loaded the MP with a solution containing 2 μM calcein-AM in HBS and delivered this solution for 2 min into a solution containing 4 mM $K_4[Fe(CN)_6]$ in HBS. During calcein-AM delivery, the feedback current was set at 80% of the initial current. Distance between the target cell and the nearest different cell was about 100 μm, and the MP was placed 150 μm apart from the target cell. The target cells were incubated for 10 min after the delivery of calcein-AM.

2.5. Induction of cellular apoptosis and monitoring with SECM-MP

We delivered an STS solution to HepG2 cells and observed cellular responses. STS is a general kinase inhibitor that blocks the binding of ATP to kinase [3,24,25]. Exposure to STS results in a significant morphological volume change because of volume-regulatory Cl^- channel activation [3]. We loaded an MP with 10 μM STS in HBS and delivered it into a solution containing 4 mM $K_4[Fe(CN)_6]$ in HBS. We chose a single spherically shaped cell as a target and positioned the MP, microelectrode, and target cell in the measurement configuration. The SECM-MP measurement was carried out automatically by the following procedure. First, cellular height was measured by time-lapse SECM measurement. Current profile measurements of the approach curve on the substrate and the cell center provided qualitative analysis of the observed cellular height [19]. Second, the STS solution was

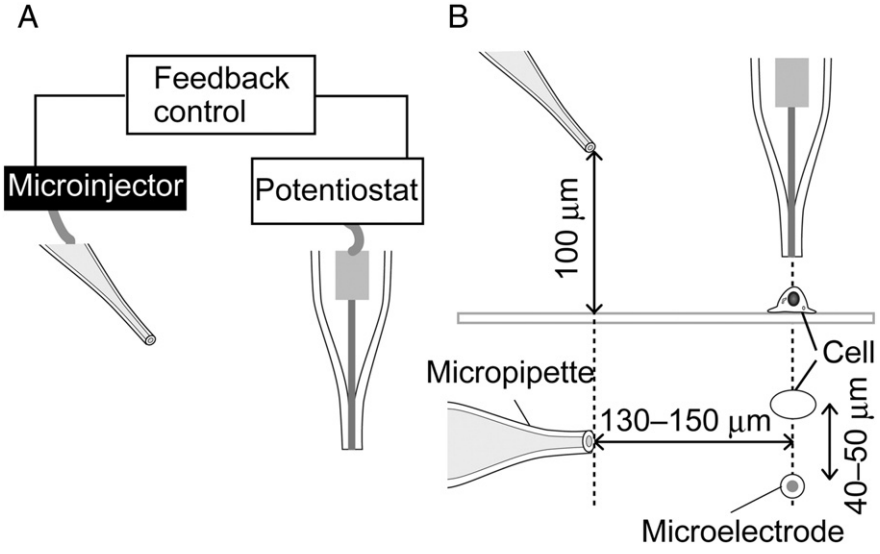

Fig. 1. Schematic illustration of the SECM-based MP system. (A) Feedback control of MP delivery using the SECM-detected current. (B) Arrangement of the MP, microelectrode, and target cell for MP delivery of a solution. All distances are indicated by double-headed arrows.