Microscope Slide Electrode Chamber for Nanosecond, Megavolt-Per-Meter Biological Investigations

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ABSTRACT

Nanosecond pulsed electric fields pass through the external membranes of biological cells (which have typical response times much greater than nanoseconds) and perturb fast-responding intracellular structures and processes. To enable real-time imaging and investigation of these phenomena, a microchamber with integral electrode walls and optical path for observing individual cells exposed to electric pulses was designed and fabricated utilizing photolithographic and microelectronic fabrication methods. SU-8 photoresist was patterned to form straight sidewalls 10 to 30 µm in height, and gold was deposited on the opposing walls and the top for a conductive, non-reactive electrode surface. Results from observations with the microchamber in real-time electroperturbation imaging experiments include intracellular calcium bursts and membrane phospholipid translocation. Real-time nanoelectropulse investigations with microfabricated imaging tools open new pathways for the study and engineering of biological systems.

Keywords: SU-8 photoresist, liftoff, megavolt electric field, microchamber, electroperturbation, microelectronic fabrication technology, microscopy

1 INTRODUCTION

Responses of biological systems to pulsed electric fields are diverse and highly dependent on the amplitude and duration of the field. Electropermeabilization (electroporation) technology utilizes pulses with durations in the millisecond and microsecond range for facilitated drug delivery and the introduction of genetic material into cells [1, 2]. Recently it has been demonstrated that nanosecond pulsed electric fields with amplitudes greater than 1 MV/m can cause apoptosis and other intracellular physiological changes not associated with traditional electroporation [3,4,5]. In the ultra-short high-field regime, the cytoplasmic membrane may be considered to be a capacitor with a charging time constant of 100 ns (approximate value for a typical mammalian cell). Pulses that are shorter than this time are felt inside the cell — across intracellular membranes (nucleus, mitochondria, storage vacuoles and other compartments). Under appropriate conditions nanosecond pulsed electric fields can thus penetrate the plasma membrane and perturb intracellular structures and processes with little or no effect on the external membrane. Ultra-short electric pulses, remotely controlled and delivered in precisely tailored amplitudes, durations, and patterns, provide a tool for manipulating the intracellular environment, inducing apoptosis or modulating gene expression noninvasively, without a requirement for direct contact with cells or tissues.

Electroperturbation has specific requirements for the pulse duration in nanosecond level and electric field over 1 MV/m. Real-time observations of electroperturbation phenomena, combining optical microscopy with the delivery of ultra-short, high-field electric pulses presents a challenge for pulse generator and exposure chamber design. In this paper we describe the design and fabrication of a microchamber –100 µm wide, 10 µm deep and 12,000 µm long – on a glass microscope slide using standard microelectronic technology, and the integration of the microchamber with an advanced technology, compact pulse generator.

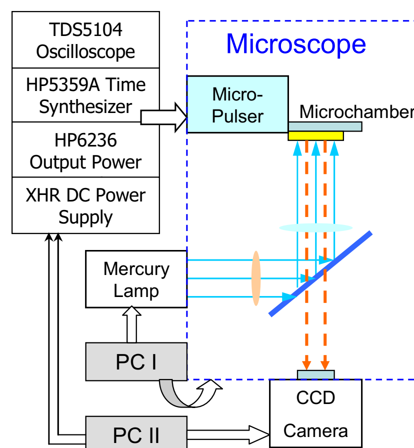


Figure 1: Real-time electroperturbation microscopy system.

2 MICROCHAMBER DESIGN AND FABRICATION

The microchamber is an integral component of a real-time electroperturbation imaging system consisting of a Zeiss Axiovert 200 microscope, a compact pulse generator designed and constructed at USC [6] and a LaVision Imager QE camera. The system diagram is shown in Figure 1.

2.1 Structure Design

Five specific material and structural requirements were identified. They are depicted in the diagram of a cross-section of an ideal channel in Figure 2.

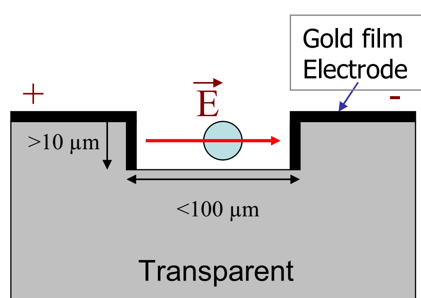


Figure 2: Ideal microchamber cross section.

1) Since the microchamber is designed for the optical study of living cells with fluorescence microscopy, the channel must be transparent to visible and ultraviolet light and may not fluoresce under excitation wavelengths from ultraviolet to infrared. To meet this condition a standard glass microscope slide was used as the substrate of the chamber. 2) The physical dimensions must accommodate the cell types under investigation, with an interelectrode distance appropriate for generating megavolt-per-meter electric fields with commercially available MOSFETs. The lymphocytes that were used in the experiments reported here have diameters up to 10 µm, so the depth of the channel must be at least 10 µm. To achieve fields up to 4 MV/m with the USC MicroPulser (400 V maximum), which is mounted along with the microchamber on the stage of an inverted microscope in a standard slide holder, the interelectrode distance must be 100 µm or less. 3) In order to provide a non-corroding conductive surface that is unreactive with biological buffers and growth media and harmless to cells, gold was chosen as the electrode material. 4) To form the smooth channel sidewalls that are perpendicular to the chamber floor, required for delivering a uniform electric field to all cells in the channel, SU-8 photoresist from MicroChem was used to build up the walls of the microchamber. 5) The length of channel is longer than 1 cm to permit the observation of reasonable numbers of living, metabolizing cells. In order to improve operational efficiency and provide more cells for experiments, four separated channels were patterned on a glass slide. The pattern is shown in Figure 3. Each channel is 12000 µm x 100 µm x 10 to 30 µm. Electrical signals may be switched independently to each channel.

Figure 3: Design schematic of the microchamber.

2.2 Fabrication

The microchamber is constructed with microelectronic fabrication technology. The key requirement of the straight sidewall was achieved by using a special material – SU-8 photoresist [7]. SU-8 is a chemically amplified, epoxy-based, negative resist. Standard formulations are offered to cover a wide range of film thicknesses from 1 to 200 µm. SU-8 has a high optical transparency and is sensitive to near UV radiation. High-aspect-ratio structures with straight sidewalls are readily formed by contact-proximity or projection printing. Cured SU-8 is highly resistant to solvents, acids, and bases and has excellent thermal stability, making it well suited for applications in which cured structures are a permanent part of the device. A double-layer photoresist liftoff step was developed to achieve satisfactory gold coverage of the channel tops and sidewalls (Figure 4).

All processing was carried out on the microscope slide substrate, which required pre-treatment to provide the smooth, clean surface needed for the foundation of the microchamber. Following a rinse with acetone and methanol, and drying with nitrogen, the slides were etched in buffered oxide etchant (10:1) for 10 minutes at room temperature, and then rinsed in DI water and acetone again. Before the lithography, samples were baked at 120 °C for at least 20 minutes. The special chemical and mechanical properties of SU-8 thick films necessitate longer exposure and development times and lower bake temperatures than those for normal photoresists. For 12 µm high channels SU-8 5 was spun on the substrate at 1000 rpm for 30 s and exposed for 35 s at 3 mW/cm^2, 365 nm. For 30 µm channels the SU-8 50 formulation was used as follows: 4000 rpm spin for 30 s, 54 s exposure at 3 mW/cm^2, 365 nm. Sharply defined edges and nearly vertical sidewalls were produced in this manner.

Because the sharp, vertical and high sidewalls were not sufficiently protected by regular photoresist in later wet etching steps, a special liftoff process was used to remove the deposited Au/Ti film in the areas outside the electrodes seen in figure 4. Before the metal film deposition, the

positive photoresist AZ5214 (1 μm thickness) was developed for liftoff with the same mask that was used for the SU-8 (negative).

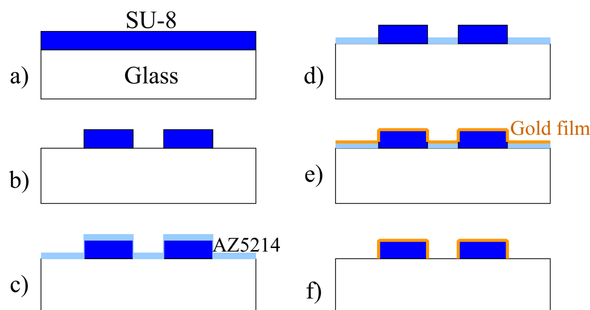


Figure 4: Double-layer photoresist liftoff processes.

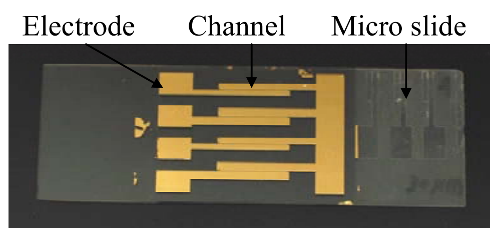


Figure 5: Microchamber on a standard microscope glass slide.

Before gold film deposition, a 50 nm Ti film was deposited on the surface of the glass substrate and photoresist channel in order to improve gold adhesion. To cover the channel sidewalls two-layer Ti/Au films were deposited with +45˚ and -45˚ angle with respect to the target source instead of the parallel position. The glass slides were rotated 90 degrees between the two steps. Different thicknesses of gold electrode from 100 nm to 250 nm were tested in experiments. The thicker film made the liftoff more difficult but improved the mechanical reliability of the microchamber in the biological experiments.

Samples were immersed in acetone for 15 to 30 minutes to lift off the metal film in the areas outside the electrodes. Ultrasonic treatment was used to accelerate the pattern forming and clean the edge in the last 5 minutes. The final microchamber pattern is shown in Figure 5.

3 EXPERIMENTAL

3.1 Cell Culture

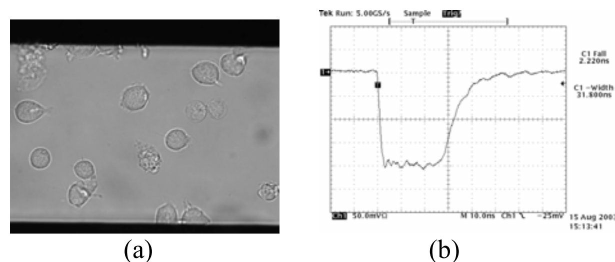
Human Jurkat T lymphocytes (ATCC TIB-152) were grown in RPMI 1640 (Irvine Scientific) containing 10% heat-inactivated fetal bovine serum (Irvine Scientific), 2 mM Lglutamine (Gibco-BRL), 50 units/mL penicillin (Gibco-BRL), and 50 μg/mL streptomycin. Live cells stained with calcium indicator Calcium Green (Molecular Probes), which exhibits a strong increase in fluorescence emission (531 nm) intensity upon binding to calcium, were monitored to observe the response of cells to the nanosecond electric pulses.

3.2 Test System

For real-time microscopic investigation, the microchamber was loaded with cells, mounted on the microscope stage in ambient atmosphere at room temperature, and electrically mated with the USC MicroPulser. The MicroPulser is a compact, solid-state pulse generator designed to provide nanosecond pulses with amplitudes variable up to 400 V into the microchamber load (200 to 400 ohms). With an interelectrode distance of 100 μm the electric field can reach 4.0 MV/m. The MicroPulser-microchamber assembly is mounted on a standard microscope stage frame so that the cells in the exposure chamber can be accessed optically and mechanically like any field on a standard slide. Images were recorded by the LaVision Imager QE CCD camera with high sensitivity working at -12 ˚C, controlled by image acquisition and analysis software DaVis by LaVision.

4 RESULTS AND DISCUSSION

An example of real-time observation of electroperturbation is shown in Figure 6 for Jurkat T cells in the 100 μm x 30 μm microchamber, a representative image from a dose-response experiment with 30 ns pulses at fields from 0.5 MV/m to 3.5 MV/m. A series of electroperturbation experiments have been conducted with the micropulser microscopy system. Among the most interesting observations have been the intracellular calcium bursts induced by nanosecond pulse exposure, visualized in cells loaded with the fluorescent calcium indicator Calcium Green. Calcium Green-stained cells exhibit a uniform and rapid release of calcium throughout the cell within milliseconds of the leading pulse edge. The plot in Figure 7 shows the fluorescence intensity changes in several cells after exposure to 30 ns 2.5 MV/m pulses. This set of curves represents 5 cells with Calcium Green fluorescence intensity increases from 8.5% to 40% after a single pulse. EGTA, a calcium chelating agent, in the external medium has no effect on calcium bursts, indicating that the source of the calcium causing the fluorescence intensity increase is



(a) (b)

Figure 6: (a) White light image of cells inside a 100 μm x 30 μm channel, (b) 30 ns pulse applied to a cell suspension in the microchamber.

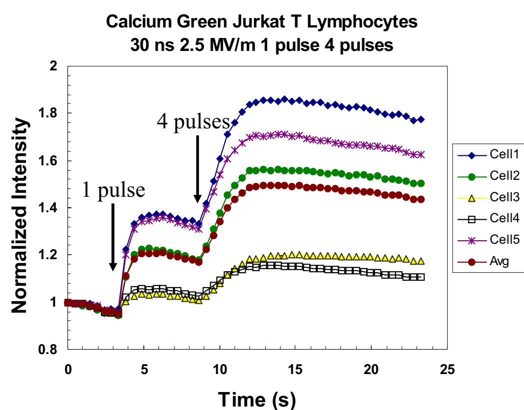


Figure 7: Fluorescence intensity change of Calcium Green after 30 ns 2.5 MV/m electric pulses.

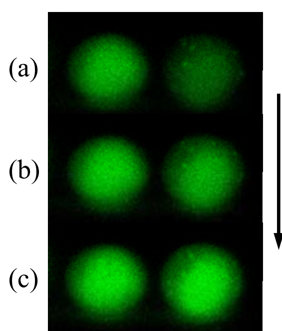


Figure 8: Fluorescent images of Jurkat T Lymphocytes stained with Calcium Green during 30 ns 2.5 MV/m electric pulses (a) cell before the shock, (b) 2 seconds after 1 pulse, (c) 4 seconds after 4 pulses.

inside the cell. The intensity change is strongly inhibited after incubation with thapsigargin, which blocks the normal filling of intracellular calcium compartments. These two sets of observations support the hypothesis that nanosecond pulses affect intracellular structures and processes (thapsigargin-inhibited calcium bursts) inside cell and not the plasma membrane (no influence from extracellular calcium).

During hundreds of hours of service some properties of the microchamber have been observed. 1) SU-8 5 photoresist has better long-term adhesion than SU-8 50 in aqueous environments. Hard-bake processing can effectively improve adhesion property, but it may cause the deformation of a large photoresist pattern due to thermal expansion, especially at high bake temperatures. 2) Weak fluorescence from SU-8 was recorded in a broad range from 450 to 600 nm. Since most areas of the channel are covered by gold film, this does not significantly affect imaging.

Further improvements may include the addition of microfluidic connections for better control of the entry and exit of cells from the channel, regulation of temperature and amosphere, and development of increased integration of the instrumentation, with full software control of pulse generation and microscope and imaging operations. The microfabricated imaging tools are opening new pathways for the investigation of biological systems and biological engineering.

5 ACKNOWLEDGEMENTS

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