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Micro-Perfusion Flow Cell for Imaging Cultured Cells

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ABSTRACT

We present a unique design for a flow cell with a small working volume that allows rapid displacement of media viewed under high power and short working distance objectives. The flow cell has a small internal depth (ca. 0.033 cm) and volume (ca. 0.05 mL) and is easy to handle. Made of Delrin$^{\dot{O}}$, the flow cell is biologically inert. We have used the flow cell for fluorescence imaging of PC12 cells loaded with tetramethylrhodamine dextran (TMRD) and other dyes.

INTRODUCTION

With the advent of new fluorescent probes, it is now possible to detect the presence and measure the concentrations of various intracellular agents and phenomena in vivo. Fluorescent probes exist for many different biologically important ions, such as Ca^{2+} (9,12), Mg^{2+} (11), Na^{+} (8), Cl^{-} (14), for intracellular pH (13) and more recently, for cAMP (1,2). These probes provide nondestructive assays that lead to gainful insights into the spatial and temporal distributions of chemical or electrical activity in living cells.

Because of the thickness and autofluorescence of many plastic substrates, it is advantageous to use glass coverslips for high-resolution fluorescence microscopy (5). Perhaps the greatest advantage to using a flow cell with a small working volume is that the technique enables a rapid medium exchange in a controlled manner, which allows cultured cells to be exposed to different stimuli in the form of pulses while quantifying the responses in real time. Berg and Block (4) presented a design for such a flow cell and used it to image bacterial flagellum movement. An identical design was used by Hernandez et al. (7) for quantifying resting-membrane potential in cultured mouse neuroblastoma N1E-115 cells.

In the Berg and Block (4) design, the cells are cultured on a 12-mm-diameter round glass coverslip that is fixed to the flow cell with a thin silicone grease film. The other window of the working chamber is a 22-mm-diameter round cover glass that is semipermanently fixed to the flow cell with silicone cement. The medium flows between the two cover glasses, allowing real-time imaging of cells (7).

The unique flow cell design reported in this article eliminates the two systemic problems encountered with the Berg and Block (4) design: *(i)* leakage from the 12-mm coverslip and *(ii)* clogging of the outlet pipe by silicone grease. In our hands, these problems reduced the number of coverslips we could analyze per day to approximately 2–3 because of the excessive cleaning required after each successful or unsuccessful attempt.

This paper describes the new flow cell designed and used for the confocal

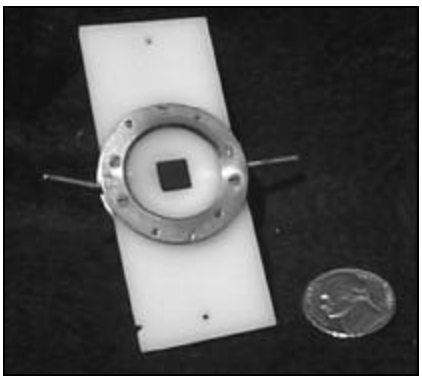

Figure 1. Photograph of the assembled flow cell.

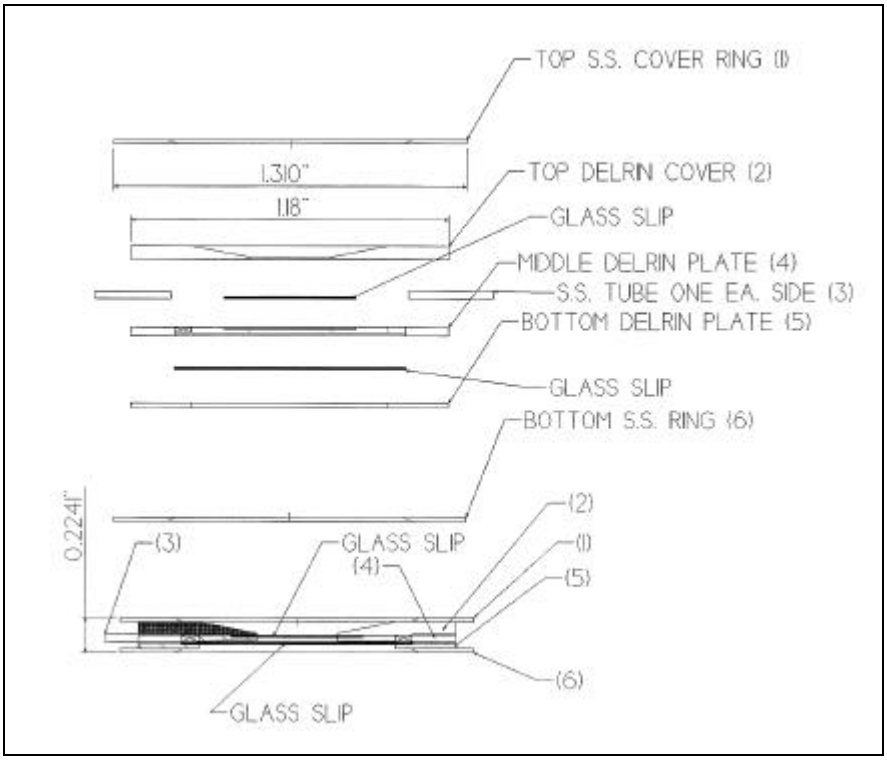

Figure 2. A side view of the flow cell, and a blowup, showing the complete assembly.

imaging of rat pheochromocytoma PC12 cells loaded with tetramethylrhodamine dextran (TMRD) and other probes.

MATERIALS AND METHODS

Flow Cell Design

A photograph of the assembled flow cell is shown in Figure 1. One of the inlet/outlet stainless steel pipes was slightly bent to allow easy connection to polyethylene tubing. The flow cell is made up of three plates. Two stainless steel rings are used to hold the plates together. The assembly of the flow cell is shown in Figure 2, and complete construction diagrams and instructions for assembly and loading are available at the BioTechniques Web site. (Go to **www.BioTechniques.com** and select *Online Journal*, search text for *Agnihotri*, click on title, then in *Abstract*, click on *Diagrams*.) The working volume is enclosed by two round glass coverslips. Solutions are drawn into the cell through a stainless steel pipe into a transverse channel and through a thin longitudinal channel into the center of the flow cell. The solutions leave by a symmetrical pathway.

For constant laminar flow through a rectangular channel, the pressure drop per unit length is inversely proportional to both the width of the channel and to the cube of its depth. In our design, similar to the Berg and Block design (4), the transverse channel is relatively deep (0.0546 cm), and the longitudinal channel is relatively shallow (ca. 0.0076 cm). Therefore, the pressure drop per unit length of the transverse channel is approximately 3% that of the longitudinal channel, with the transverse channel acting as a constant pressure source that feeds fluid into the longitudinal channel along its entire width. The flow cell is nearly uniform from side to side and has a volume of approximately 0.05 mL.

Cell Culture and Loading of Dye

The rat pheochromocytoma cell line PC12 was obtained from ATCC (Rockville, MD, USA) and was cultured in RPMI 1640 growth medium (Grand Island Biological, Grand Island, NY, USA), supplemented with 10% horse serum (JRH Biosciences, Lenexa, KS, USA) and 5% VSP neonate bovine serum (Biocell Laboratories, Rancho Dominguez, CA, USA) and 50 U/mL penicillin/streptomycin (Sigma, St. Louis, MO, USA) (6). Cells were exposed to 50 ng/mL 2.5S nerve growth factor (10) for at least seven days before an experiment. One to two days before an experiment, cells were subcultured onto 22-mm (or 12-mm) round glass coverslips that had previously been treated with 0.7% polyethylenimine (Sigma) and washed in tissue culture medium before use. We were more successful with 22-mm coverslips, since they allowed more room in our efforts to identify the cells of interest. Approximately 10 cells were pressure microin-

jected with TMRD, molecular weight 70 kDa (Sigma). Cells located close to the center of the coverslip were preferred and allowed easy location during microscopic examination.

Flow Cell Characterization and Use

To confirm the rapid medium exchange, we used fluorescein [in phosphate-buffered saline (PBS)] pulses lasting between 30 s to 5 min at flow rates of 279 and 733 µL/min. Fluorescein fluorescence was measured with computer-controlled DeltaScan™ System (Photon Technology International, Monmouth Junction, NJ, USA) coupled to a Model IX70 Inverted Microscope (Olympus America, Melville, NY, USA). The IX70 was fitted with an Uapo/340 40× objective, a Fura-2 Filter Set (Chroma Technology, Brattleboro, VT, USA) and Model IC100 Digital Camera (Photon Technology International). The DeltaScan was run under ImageMaster™ software control. Fluorescein was excited at 370 nm, and the emission was monitored at 520 nm.

To image TMRD, cells were microinjected with dye, coverslips were loaded into the flow cell as described, and the flow cell was immediately transferred to the stage of an Optiphot Microscope (Nikon, Melville, NY, USA) that was maintained at 37°C with an air curtain incubator (Nicholson Precision Instruments, Gaithersburg, MD, USA). Using the appropriate filter for rhodamine, injected cells were located and imaged using a Model MRC 600 Confocal Laser-Scanning Microscope (Bio-Rad, Hercules, CA, USA), equipped with a krypton/argon mixed gas laser. A 568-nm excitation cube was used for TMRD. The focal plane was selected to maximize fluorescence from the cells. To image cells, we used a Zeiss 25X 0.8 na-multi immersion objective (Carl Zeiss, Thornwood, NY, USA), and the software COMOS 6.1 was used to acquire and save the images to disk.

RESULTS AND DISCUSSION

Figure 3 shows 30 s, 1 and 5 min fluorescein pulses at 279 and 733

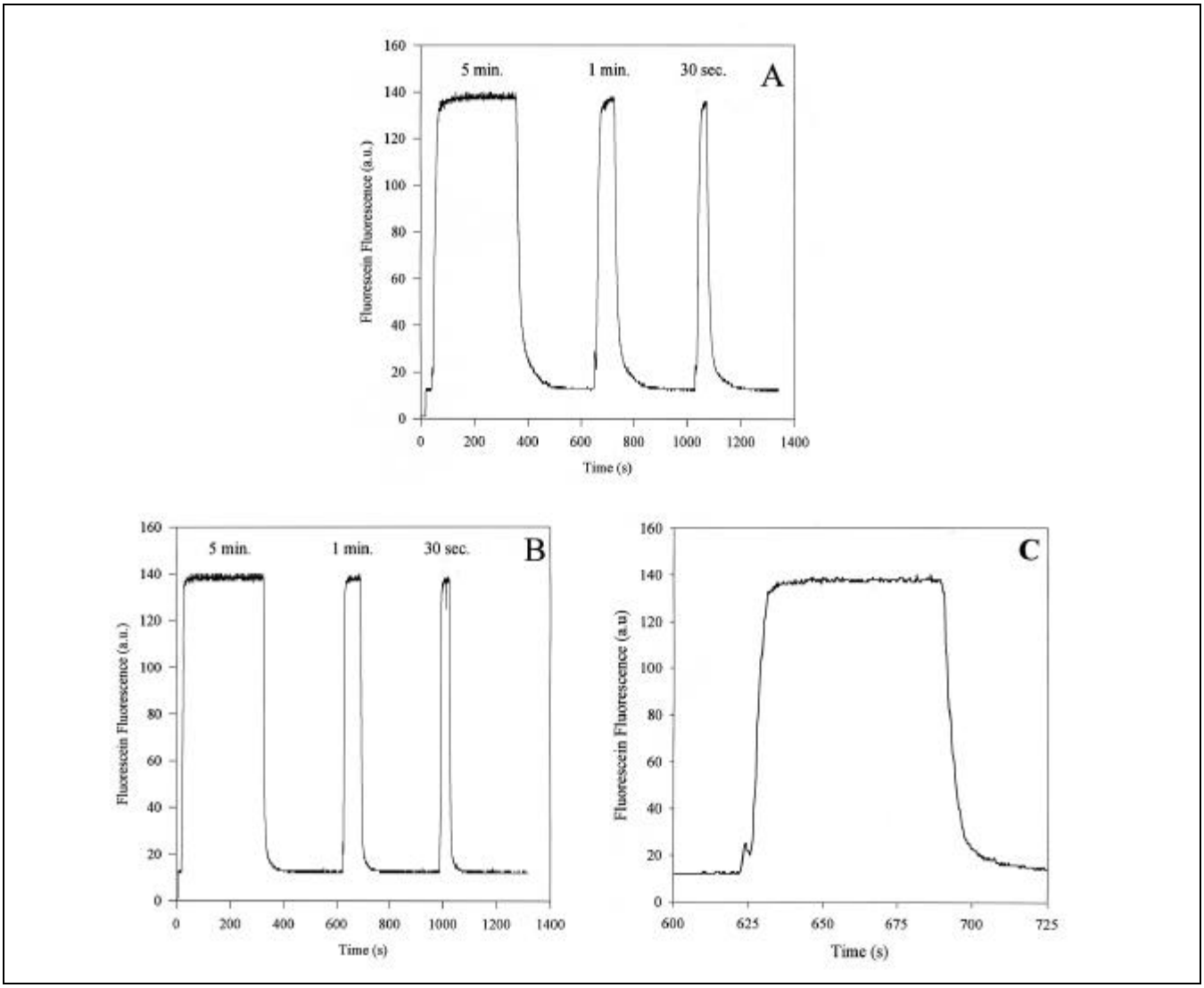

Figure 3. Fluorescein pulses at 279 (A) and 733 (B) µL/min PBS flow rates as well as the 1-min pulse in Panel B but at a larger scale (C).

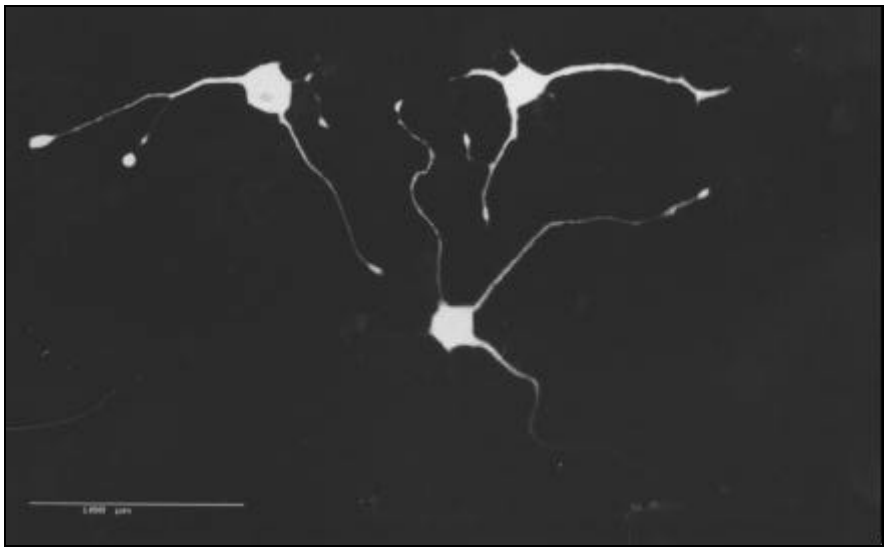

Figure 4. PC12 cells microinjected with TMRD, mol wt 70 kDa. Bar = 100 μm.

μL/min flow rates, as well as the 1-min pulse in B, but at a larger scale. As shown (Figure 3), the time required to reach 60% of the maximum fluorescein concentration was negligibly small when compared to the pulse period. Figure 4 shows PC12 cells microinjected with TMRD imaged at 568 nm. The focal plane for these images was approximately 3–9 μm below the top of the cells. Note the smooth boundaries of the cells and the well-extended neurites, indicating a lack of toxic effects. It was possible to observe and image neurites without any distortions, confirming that the flow cell design seemed to eliminate the coverslip flexing problem mentioned by Berg and Block (4). At a flow rate of 279 μL/min, the peristaltic pump generated a pulsation every 3 s. The fact that Kalman averages of 3 images every 2 s yielded no statistically significant difference between images taken over 5 min confirmed further that, under these conditions, flexing of the coverslip was negligibly small. Also, the formation of bubbles due to poor sealing and/or sub-atmospheric pressure in the flow cell was not observed with the current design.

We also used the flow cell to measure intracellular cAMP concentrations using the fluorescent probe FlCRhR, a single-excitation, dual-emission probe whose emission spectrum changes upon cAMP binding, allowing the intracellular free cAMP concentrations to be continuously monitored in space and time in living cells microinjected with the probe (1,2). Due to the small working volume, the flow cell allowed rapid displacement of medium, and thus enabled stimulation of the cultured cells with pulses. The results for cAMP imaging have been reported elsewhere (3). We used the flow cell to image fluorescein isothiocyanate (FITC) dextran and Fura-2 in mouse neuroblastoma N1E-115 and human bone osteosarcoma TE-85 cells.

The flow cell presented in this paper is made of the synthetic plastic Delrin® in three parts, held together with two stainless steel rings and six screws, providing a robust design with greater reliability. As in the Berg and Block design (4), the dimensions of the two round, glass coverslips are 12 and 22 mm diameter. Silicone grease is used only as a support while assembling the cell and not as the primary sealant, essentially eliminating the clogging problem. These improvements minimized the number of unsuccessful attempts per experiment. Using the flow cell, we have successfully worked with up to 12 coverslips in a single experiment in comparison to 2–3 coverslips with the Berg and Block design (4). Since the bottom (22 mm) coverslip is not semi-permanently fixed with cement, cells can be cultured on either one of the coverslips (12 or 22 mm), which gives the flow cell a more flexible design. Over-

all, the design provides a more controlled system with much greater reliability. In addition, because the medium bathing the cells is only in contact with the biologically inert Delrin, toxic effects on the cells are minimized.

The ability to dynamically monitor signals in living cells provides unprecedented spatial and temporal resolution in the study of the distribution of chemical and electrical activity. This flow-cell design provides a convenient means of displacing the medium bathing the cells in a controlled manner, so that various kinds of stimuli can be delivered to cells during imaging.

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Naveen Agnihotri, William S. Kisaalita and Charles H. Keith
University of Georgia
Athens, GA, USA

Co-Transfected SV40 Origin of Replication Activates Expression from SV40 Promoterless Constructs

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ABSTRACT

Co-transfection with expression plasmids is widely used to control DNA uptake efficiency in transient transfection experiments. However, a number of problems have been associated with their use. Here, we describe the activation of expression of constructs not containing the simian virus 40 (SV40) origin of replication (ori) by co-transfection in COS-7 cells with plasmids containing the SV40 ori. This effect has consequences for the use of such plasmids to control transfection efficiency.

INTRODUCTION

Where cells are transfected in separate culture dishes or wells and it is required to compare expression between cells transfected in separate batches, it is necessary to correct results for efficiency of the transfection process (1). This is usually achieved by co-transfection with a control-expression plasmid, often one expressing a conveniently measured product such as β-galactosidase (β-gal) or luciferase. Although this procedure is used widely, there are problems associated with it. For instance, there may be inhibitory effects of one plasmid on another (4), possibly as a result of competition for transcription factors or unexplained, synergistic effects between co-transfected plasmids (14). In comparing control plasmids for use with COS-7 cells, we have observed activation by plasmids containing the simian virus 40 (SV40) origin of replication (ori) of those that do not contain this sequence. This can lead to a high background of reporter gene expression. Since many commonly used control plasmids contain the SV40 ori, this is a frequently encountered but easily avoided problem.

MATERIALS AND METHODS

pCAT® Basic and pCAT Promoter Vectors were from Promega (Southampton, England, UK). pCMV5hER (11), expressing the human estrogen receptor was the gift of Prof. B.S. Katzenellenbogen (University of Illinois, Urbana-Champaign, IL, USA). pBL2CATERE, expressing chloramphenicol acetyltransferase (CAT) under the control of the vitellogenin A_2 estrogen-response element and herpes simplex virus thymidine-kinase promoter, and pMT2mER, expressing the mouse-estrogen receptor, were derived from the constructs described by Luckow and Schütz (10) and Kaufman et al. (9)