July 2008

Microfluidic electroporation for selective release of intracellular molecules at the single-cell level

Ning Bao
Purdue University, nbao@purdue.edu

Jun Wang
Purdue University, wangj@purdue.edu

Chang Lu
Birck Nanotechnology Center, Purdue University, changlu@purdue.edu

Ning Bao
Jun Wang
Chang Lu

Department of Agricultural and Biological Engineering, Bindley Bioscience Center, Birck Nanotechnology Center, Purdue University, West Lafayette, IN, USA

Received November 26, 2007
Revised January 27, 2008
Accepted March 3, 2008

Research Article

Microfluidic electroporation for selective release of intracellular molecules at the single-cell level

Analysis of intracellular materials at the single-cell level presents opportunities for probing the heterogeneity of a cell population. Lysis by electroporation has been gaining popularity as a rapid method for disruption of the cell membrane and release of intracellular contents. In this report, we selectively released specific intracellular molecules for interrogation at the single-cell level by tuning the parameters of electroporation. We examined the release of a small molecule, calcein (MW∼600), and a 72-kDa protein kinase, Syk, tagged by enhanced green fluorescent protein (EGFP) from chicken B cells during electroporation at the single-cell level. We studied the effects of the field intensity and the field duration on the release of the two molecules. We found that calcein in general was released at lower field intensities and shorter durations than did SykEGFP. By tuning the electrical parameters, we were able to deplete calcein from the cells before SykEGFP started to release. This approach potentially provides a high-throughput alternative for probing different intracellular molecules at the single-cell level compared to chemical cytometry by eliminating complete disruption of the cell membrane.

Keywords:
Cytometry / Electroporation / Intracellular molecules / High throughput / Microfluidics
DOI 10.1002/elps.200700856

1 Introduction

Studies of intracellular molecules at the single-cell level are important for probing the heterogeneity of cell populations. Flow cytometry has been the tool of choice for single-cell studies within cell populations of large sizes. The state-of-the-art flow cytometer can measure multiple intracellular molecules simultaneously up to 17 colors with the throughput to reach 50 000 cells per second [1, 2]. However, flow cytometric analysis requires complicated procedures for specific fluorescent labeling of the target molecules, which includes fixation, and permeabilization of the cells. More importantly, the researchers can only obtain knowledge about the molecules that they target. This makes some tasks such as the discovery of new biomarkers challenging in most cases. Recent advances in instrumentation and emergence of new tools such as microfluidics have allowed integrated release and analysis of intracellular molecules at the single-cell level [3–6]. Single-cell analysis based on released intracellular materials, or chemical cytometry, typically requires disruption of the cell membrane and release of the intracellular materials before the intracellular molecules are analyzed by tools such as electrophoresis. Chemical cytometry is largely limited by its throughput (<85 cells/min), due to the low speed for both cell lysis and electrophoresis [6].

In this study, we demonstrated the combination of flow-through electroporation [7] with flow cytometry for the selective release of intracellular molecules under different electroporation parameters at the single-cell level with a high throughput (∼200 cells/s). We examined the release of calcein (MW∼600) and a protein kinase, Syk (72 kDa), tagged by enhanced green fluorescent protein (EGFP) from B cells during electroporation. Protein kinases are interesting targets in a number of single-cell analysis reports [8, 9]. Syk is the prototype kinase of the Syk/Zap-70 family and is essential for the survival, proliferation and differentiation of B lymphocytes [10, 11]. Our method suggests an alternative approach for the analysis of specific intracellular molecule(s) at the single-cell level with high throughput.

Correspondence: Dr. Chang Lu, Department of Agricultural and Biological Engineering, 225 S. University Street, West Lafayette, IN 47907, USA
E-mail: changlu@purdue.edu
Fax: +1-765-496-1115

Abbreviation: **EGFP**, enhanced green fluorescent protein

2 Materials and methods

2.1 Cell culture and preparation

The creation of stably transfected chicken DT40 B cells expressing SykEGFP fusion protein was described previously [12]. Briefly, an *Xho*I/*Hpa*I DNA fragment encoding Syk was cut from EPB:SykMyc [13]. Insertion of this frag-

ment into *Xho*I/*Sma*I sites of the pEGFP-N2 vector (Clontech, Palo Alto, CA) resulted in a fusion of Syk and GFP (SykEGFP). Syk-deficient (DT40-Syk$^-$) [14] chicken DT40 B cells (1×10^6) were transfected with 20 μg of the DNA construct by electroporation [15]. DT40-Syk$^-$ and SykEGFP-DT40-Syk$^-$ cell lines were applied in this study. They were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% chicken serum, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate and 100 IU/mL penicillin G for 10–15 passages before the experiments. In the experiment, the cells were harvested and then centrifuged at 300 rpm for 10 min. The cells were then resuspended in an electroporation buffer containing 10 mM HEPES and 250 mM sucrose (pH 7.4, autoclaved). Calcein AM loading of DT40-Syk$^-$ cells or SykEGFP-DT40-Syk$^-$ cells was carried out by incubating the cells in the electroporation buffer with 0.003 μg/mL calcein AM in the dark for 20 min. The cells have a density of around 10^7/mL in the electroporation buffer before the experiments.

2.2 Microfluidic chip fabrication

PDMS-based microfluidic devices were fabricated using standard soft lithography method as described before [6, 7]. The microchannel structures were first designed using the FreeHand MX (Macromedia, San Francisco, CA) software and then printed out on the transparencies with the resolution of 5080 dpi. The transparencies were then used as the photomasks in photolithography for the fabrication of relief masters on silica wafer using a negative photoresist SU-8 2025 (MicroChem, Newton, MA). The photoresist was coated using a spin coater at 3000 rpm for 30 s. The thickness of the photoresist (also the depth of the microchannels) was measured to be 2 5 $\pm$ 1 μm by a Sloan Dektak3 ST profilometer. The PDMS (General Electric Silicones RTV 615, MG chemicals, Toronto, Ontario, Canada) prepolymer mixture (monomer A:curing agent B = 10:1, mass ratio) was cast on the master and cured at the temperature of 80°C for 2 h. Glass slides were cleaned in a basic solution (H_2O:NH_4OH(27%):H_2O_2 (30%) = 5:1:1, volumetric ratio) at 75°C for 1 h and then rinsed with deionized water and blown dry. The PDMS chip (peeled off from the master and punched to form access holes) and a glass slide were oxidized using a Tesla coil (Kimble/Kontes, Vineland, NJ) in air and then immediately brought into contact to form closed devices. Teflon tubing (1622L, Upchurch Scientific, Oak Harbor, WA) was connected to the inlets of the channels for the transport of the cell sample and buffers.

2.3 Microchip operation and data acquisition

The microfluidic chip was mounted on an inverted fluorescence microscope (IX-71, Olympus, Melville, NY) with a 60X dry objective (NA = 0.70). Prior to the experiment, the microfluidic channel was flushed with the electroporation buffer for 15 min to condition the channel and remove impurities. The three inlets of the channel were connected to a syringe pump (PHD infusion pump, Harvard Apparatus, Holliston, MA) through plastic tubing. The volumetric flow rates were set at 1 μL/min for the sample channel inlet and 5 μL/min for each of two side channel inlets. With a cell density of 10^7/mL, ~200 cells flowed through the laser detection spot per second. A high-voltage power supply (PS350, Stanford Research Systems, Sunnyvale, CA) was used to generate a constant direct current (DC) voltage in between the sample and the outlet reservoirs for electroporation. Green fluorescent microspheres with the diameters of 10 and 1 μm (Duke Scientific, Fremont, CA) were also applied for the validation of the system. An air-cooled argon ion laser (50 mW, Spectra-Physics, Mountain View, CA) at 488 nm was applied as the excitation source for LIF. The laser beam was spectrally filtered by a 10LF10-488 band-pass filter (Newport, Irvine, CA) before its intensity was adjusted by neutral density filters (Newport). The laser was introduced into the microscope through laser port (Olympus) and a fluorescence filter cube consisting of 505DCLP dichroic beam splitter and D535/40 emission filter (Chroma Technology, Rockingham, VT) before it was finally focused by the objective into the microfluidic channel. The intensity of the laser coming out of the objective was ~ 3 mW. The emission light was collected by the same objective and converted into current by a R9220 photomultiplier tube (Hamamatsu, Bridgewater, NJ) biased at –450 V. The photocurrent was amplified and filtered by a SR570 low-noise current preamplifier (Standard Research System) with the parameters set at low-noise mode, low-pass filter, 100-kHz cutoff frequency. The current was then converted to be voltage and digitalized by the PCI-6254 data acquisition card (National Instrument, Austin, TX) in a PC. A LabView program was used to record the digitalized signal at the frequency of 10^6 Hz. The data were processed by programs written in MATLAB to extract histograms of the fluorescence for a cell/bead population. The data were presented in 4-decade (from 0.001 to 10 V) logarithmic histograms with 256 channels. The voltage signal ranging from 1 mV to 10 V was converted to 4-decade logarithmic voltage scale and then 256 channels [16]. Each histogram contained data collected from 2000–3000 cells.

3 Results and discussion

The electroporative release of intracellular materials can be conveniently monitored at the single-cell level by combining a microfluidic electroporation technique we demonstrated previously [7, 17] with flow cytometric detection. As shown in Fig. 1, the design of the microfluidic device is similar to the one used in our previous study [18]. The device had a horizontal channel with varying widths. The horizontal channel intersected with a vertical channel. A constant dc voltage was applied across the horizontal channel during our experiment. The field intensity in the narrow section was much higher than that in the wide sections in the horizontal chan-

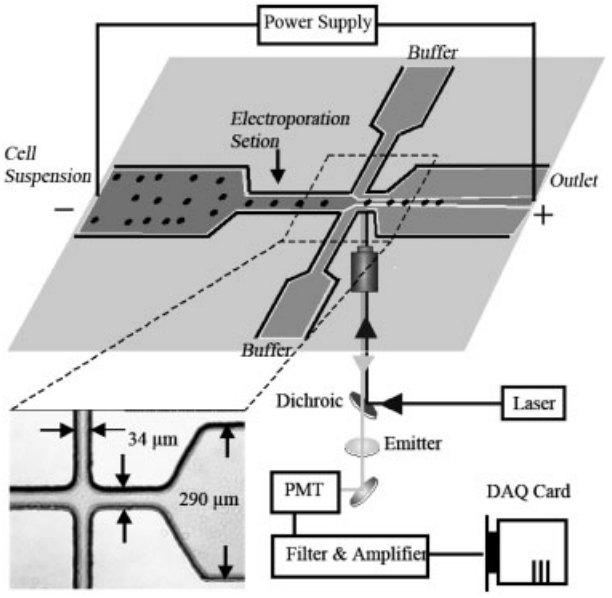

Figure 1. Schematic of the microfluidic system for the electroporative release of intracellular materials at the single-cell level. The details about the setup and the data collection are given in Section 2.

nel with the ratio in the field intensity being inversely proportional to the ratio in the width (with the width of 34 μm of the narrow channel and the width of 290 μm of the wide channel, the field intensity was around 8.5 times higher in the narrow section than in the wide sections) [7]. Electroporation occurred only in the narrow section of the horizontal channel when the overall voltage applied was in the right range. The low field intensity in the reservoirs and wide sections did not cause electroporation or release of intracellular materials as we had demonstrated before [7]. The vertical channel provided two side streams to hydrodynamically focus the cells to the center of the horizontal channel so that they could be detected in the laser focal volume at the exit of the narrow section (the electroporation section) at the single-cell level, similar to the practice in flow cytometry. We were able to measure how much of the fluorescent materials (calcein, SykEGFP, or both) was released due to electroporation by comparing the fluorescence histogram of a cell population after electroporation with that of the control population (without the field). The unique flow-through feature of this electroporation technique allowed a seamless integration with the flow cytometry. The field intensity in the narrow section was determined by the geometry of the channel and the total voltage applied. The duration of the electroporation could be calculated based on the velocity of the cells and the length of the narrow section. We found that the cell velocity did not substantially change with the field intensity which suggested that the electrophoretic mobility of cells was trivial compared to the overall velocity. Therefore, the duration of the electroporation could be calculated based on the infusion rate of the syringe pump and the dimensions of the narrow section. We used three different lengths (1, 2, and 3 mm) for the narrow section in different device layouts. With the depth of 25 μm and the width of 34 μm in the narrow section, these lengths of the narrow section provided field durations of ~50, 100 and 150 ms, respectively, when the flow rates of the cell sample and two focusing streams were set at 1, 5 and 5 μL/min, respectively. When the field intensity was 0 (no voltage applied across the horizontal channel), the device worked as a microfluidic flow cytometer.

To validate the system for detecting the fluorescent intensity of single particles, we applied the system to screen a mixture of fluorescent microspheres with diameters of 10 and 1 μm (Fig. 2). Due to the different numbers of particles scanned in every run, we used percentile counts (counts/total counts of the run) to facilitate comparison among histograms. The integral of each histogram was equal to 1. The fluorescent intensity was converted to channels (0–255) following the procedure described in Section 2. The average fluorescent intensity generated by 1-μm beads was 75, 76, 77 and 79 channels at the field intensity of 0,400, 800, 1200 V/cm, respectively. The average fluorescent intensity generated by 10-μm beads was 183, 184, 183 and 184 channels at 0, 400, 800, 1200 V/cm, respectively. This indicates that the application of the field does not vary the velocity and the detected intensity of the microspheres in a substantial way especially for large beads with their size close to that of cells. The setup was effective in producing fluorescence histograms reflecting the distribution in a population of particles.

A small molecule, calcein AM, was loaded into chicken DT40 cells without SykEGFP fusion protein (DT40-Syk$^-$) for the investigation of the release of its fluorescent product, calcein, due to electroporation. Calcein AM is a fluorogenic dye, which is converted to fluorescent calcein by esterases once inside the cells. Calcein is membrane-impermeant and well-retained within the membrane. Figure 3A shows a raw spectrum of fluorescent intensity over time recorded by the photomultiplier tube. The spikes were generated by individual cells flowing through the detection point. The spectrum indicates that the throughput of cells was ~200 cells/s. Figure 3B was extracted from the raw data and shows the fluorescent intensity histograms generated by calcein AM-loaded cell populations after electroporation under different electric field intensities and durations. The field intensity was varied by adjusting the voltage and the field duration was changed by using the devices with different lengths for the narrow section while keeping the flow rates fixed. Since the fluorescent intensity was detected at the exit of the narrow section, the intensity reflected the fluorescent material remaining in the cells at the end of the electroporation process. When compared to the control taken without the field (0 V/cm), there was no significant decrease in the fluorescent intensity of the histogram until the field intensity increased beyond

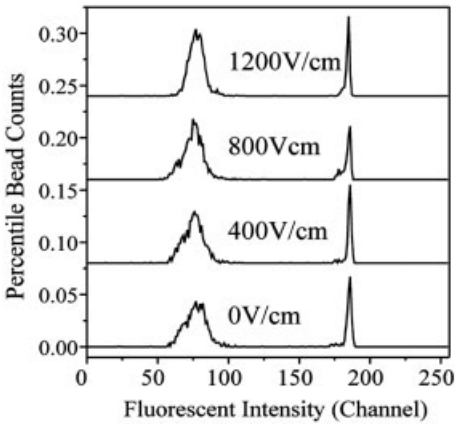

Figure 2. Histograms of fluorescent intensity generated by mixed microspheres (D~10 and 1 μm) under different electric field intensities in the narrow section taken using the device in Fig. 1 in the main text.

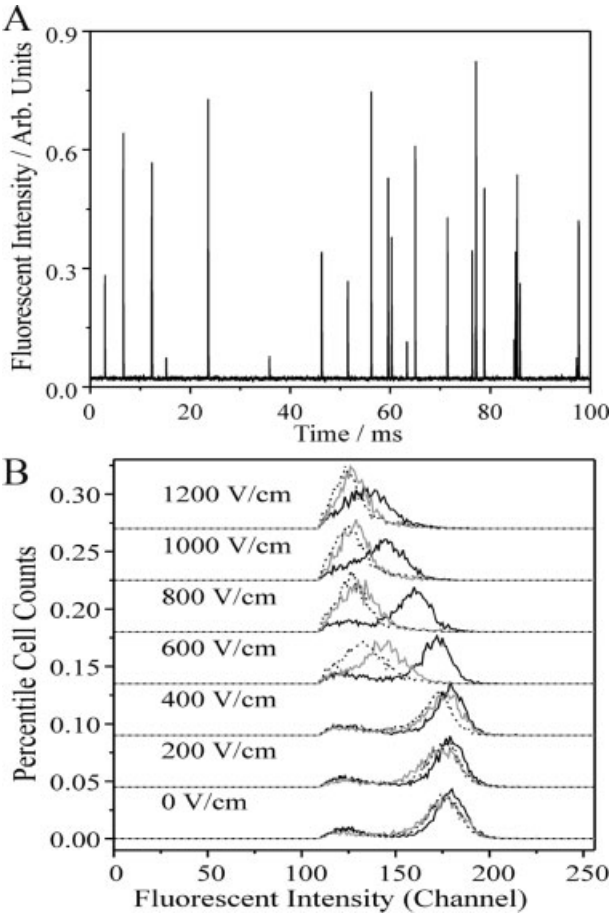

Figure 3. (A) Fluorescent intensity over time recorded by the photomultiplier tube when calcein AM-loaded DT40-Syk$^-$ cells flow through the device. (B) Histograms of fluorescent intensity generated by calcein AM-loaded DT40-Syk$^-$ cells under different electric field intensities and different durations (black, 50 ms; gray, 100 ms; dot line, 150 ms).

400 V/cm. In general, higher field intensity and longer field duration shift the intensity distribution to the lower end. The field duration had substantial effect on the fluorescent intensity of the cell population when the field intensity was beyond 400 V/cm. This effect is the most obvious at 600 V/cm as the average fluorescent intensity shift from 161 (SD $\sigma = 18$, assuming normal distribution) to 142 ($\sigma = 18$) and 134 ($\sigma = 19$), when the field duration was increased from 50 to 100 and 150 ms, respectively. With the field intensity between 600 and 1200 V/cm, the average fluorescent intensity of the cell population with a field duration of 50 ms was always substantially higher than those of 100 and 150 ms. The fact that the release of calcein was dependent on the field duration in the range of 50–150 ms indicates that the release occurred at the time scale of 50–150 ms when the field was in the range of 600–1200 V/cm.

In Fig. 4, we show the phase contrast and fluorescent images of calcein AM-loaded cells after experiencing 150-ms electroporation duration at various field intensities. The fluorescent intensity of cells decreased as the result of electroporative release. The fluorescent intensity decreased more when the field intensity was higher. The majority of the fluorescence was gone at 600 V/cm and this agrees with the

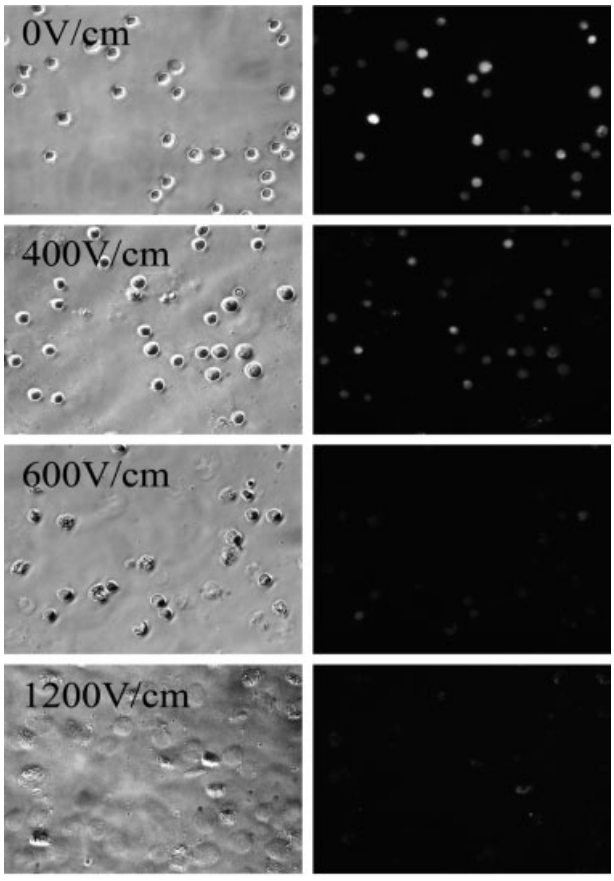

Figure 4. Phase contrast (left panel) and fluorescent (right panel) images of calcein AM loaded cells after being processed by the device with a field duration of 150 ms at various field intensities (0, 400, 600, and 1200 V/cm).

results in Fig. 3B. The phase contrast images show that the cells appeared flat and empty with a substantial fraction of the intracellular contents gone when the field intensity was very high at 1200 V/cm.

Figure 5 shows the release of SykEGFP fusion protein from SykEGFP-DT40-Syk$^-$ cells under various electrical parameters. The change in the histogram with various field intensities and durations for SykEGFP release is quite different from that for calcein release. As demonstrated in Fig. 3B, the calcein intensity histogram had significant shift to the lower fluorescence due to release when the field intensity was 600 V/cm especially with 100- and 150-ms durations. In comparison, SykEGFP was not released significantly with any field duration until the field intensity reached 800 V/cm (as shown in Fig. 5). It has been suggested the higher field intensity creates larger pores in the membrane [19]. Since the molecular size of SkyEGFP is significantly larger than that of calcein, this explains why SykEGFP requires higher field intensity for the electroporative release than calcein.

When the cells contained both calcein and SykEGFP (calcein AM-loaded SykEGFP-DT40-Syk$^-$ cells), we were able to selectively release calcein first without releasing SykEGFP by tuning the electrical parameters. As shown in Fig. 6, the histogram generated by the cell population containing both SykEGFP and calcein located at substantially higher fluorescent intensity than that of the cell population containing only SykEGFP, when the examination was taken at 0, 200 and 400 V/cm. However, the two histograms overlapped when the field intensity was increased to 600 V/cm and beyond since calcein was depleted from the cells at this field intensity and duration before the release of SykEGFP started at 800 V/cm. In this case, the selective release of intracellular molecules allows us to selectively release one molecule at a time at the single-cell level by tuning the electrical parameters of electroporation. When combined with a proper detection method, the released molecule can be potentially analyzed at the single-cell level as demonstrated in previous work [20, 21] and the different molecules can be analyzed under different operational conditions. Compared to the alternative of using chemical cytometry (complete lysis of single cells followed by electrophoresis of the cell lysate) with a throughput of 85 cells/min or less [4, 6], our approach currently offers a throughput of ~200 cells/s and potentially yields a throughput comparable to that of flow cytometry (~10^4 cells/s) due to the elimination of complete cell lysis.

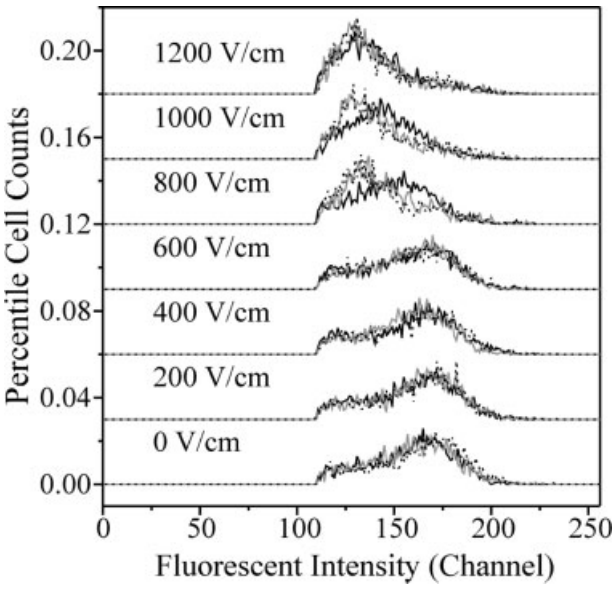

Figure 5. Histograms of fluorescent intensity generated by SykEGFP-DT40-Syk$^-$ cells under different electric field intensities and different durations (black, 50 ms; gray, 100 ms; dot line, 150 ms).

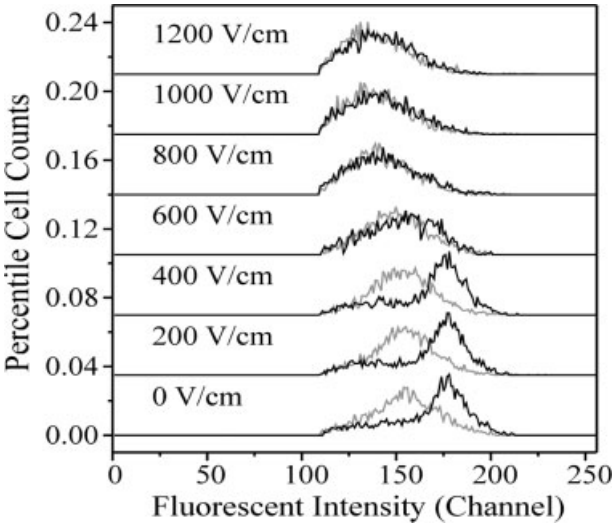

Figure 6. Comparison between the histograms of fluorescent intensity generated by calcein AM-loaded SykEGFP-DT40-Syk$^-$ cells (black) and those generated by SykEGFP-DT40-Syk$^-$ cells (gray) under different electric field intensities with the duration of 100 ms.

4 Concluding remarks

We demonstrated the selective release of intracellular molecules (calcein and SykEGFP) at the single-cell level by tuning the parameters of flow-through electroporation. The release of intracellular molecules into the surrounding solution during electroporation is likely related to the molecules' physical properties such as diffusivity and electrophoretic mobility. Furthermore, the molecule size relative to that of the electropores generated in the membrane also determines how difficult the release process is. Our results showed that the electroporative release of a certain intracellular molecule required a set of threshold conditions. Only electroporation

with higher intensity and longer duration than the threshold ones releases the molecule. The threshold conditions for electroporative release of different intracellular molecules vary substantially, depending on their physical characteristics. The electroporative release provides a unique way for differentiating the intracellular molecules in our approach. By combining flow-through electroporation with flow cytometry, we proposed a unique and high-throughput approach to study specific intracellular molecule(s) at the single-cell level.

The authors acknowledge partial support through a cooperative agreement with the Agricultural Research Service of the US Department of Agriculture, project number 1935-42000-035 through the Center for Food Safety Engineering at Purdue University. We also thank Dr. Robert Geahlen for providing the chicken B cell lines.

The authors declared no conflict of interest.

5 References

[1] Ibrahim, S. F., van den Engh, G., *Curr. Opin. Biotechnol.* 2003, *14*, 5–12.

[2] Perfetto, S. P., Chattopadhyay, P. K., Roederer, M., *Nat. Rev. Immunol.* 2004, *4*, 648–655.

[3] Krylov, S. N., Starke, D. A., Arriaga, E. A., Zhang, Z. *et al.*, *Anal. Chem.* 2000, *72*, 872–877.

[4] McClain, M. A., Culbertson, C. T., Jacobson, S. C., Allbritton, N. L. *et al.*, *Anal. Chem.* 2003, *75*, 5646–5655.

[5] Wu, H., Wheeler, A., Zare, R. N., *Proc. Natl. Acad. Sci. USA* 2004, *101*, 12809–12813.

[6] Wang, H. Y., Lu, C., *Chem. Commun.* 2006, 3528–3530.

[7] Wang, H. Y., Lu, C., *Anal. Chem.* 2006, *78*, 5158–5164.

[8] Meredith, G. D., Sims, C. E., Soughayer, J. S., Allbritton, N. L., *Nat. Biotechnol.* 2000, *18*, 309–312.

[9] Sims, C. E., Allbritton, N. L., *Curr. Opin. Biotechnol.* 2003, *14*, 23–28.

[10] Zioncheck, T. F., Harrison, M. L., Geahlen, R. L., *J. Biol. Chem.* 1986, *261*, 15637–15643.

[11] Turner, M., Mee, P. J., Costello, P. S., Williams, O. *et al.*, *Nature* 1995, *378*, 298–302.

[12] Ma, H., Yankee, T. M., Hu, J., Asai, D. J. *et al.*, *J. Immunol.* 2001, *166*, 1507–1516.

[13] Keshvara, L. M., Isaacson, C., Harrison, M. L., Geahlen, R. L., *J. Biol. Chem.* 1997, *272*, 10377–10381.

[14] Takata, M., Kurosaki, T., *J. Exp. Med.* 1996, *184*, 31–40.

[15] Keshvara, L. M., Isaacson, C. C., Yankee, T. M., Sarac, R. *et al.*, *J. Immunol.* 1998, *161*, 5276–5283.

[16] Shapiro, H. M., *Practical Flow Cytometry*, John Wiley & Sons, Hoboken, New Jersey 2003.

[17] Wang, H. Y., Lu, C., *Biotechnol. Bioeng.* 2006, *95*, 1116–1125.

[18] Wang, J., Bao, N., Paris, L. L., Wang, H. Y. *et al.*, *Anal. Chem.* 2008, *80*, 1087–1093.

[19] Benz, R., Beckers, F., Zimmermann, U., *J. Membr. Biol.* 1979, *48*, 181–204.

[20] Gao, N., Wang, W., Zhang, X., Jin, W. *et al.*, *Anal. Chem.* 2006, *78*, 3213–3220.

[21] Gao, N., Zhao, M., Zhang, X., Jin, W., *Anal. Chem.* 2006, *78*, 231–238.

