ELECTROPORATION-BASED SELECTIVE EXTRACTION OF SUBCELLULAR PROTEINS

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ABSTRACT

Conventional biochemical analysis mainly focuses on the expression level of cellular proteins from entire cells. However, it has been increasingly acknowledged that the subcellular location of proteins often carries important information. Analysis of subcellular proteins conventionally requires subcellular fractionation which involves two steps: cell lysis to release proteins and high-speed centrifugation to separate the homogenate. Such approach requires bulky and expensive equipment and is not compatible with processing scarce cell samples of limited volume. In this study, we apply microfluidic flow-through electroporation to breach cell membranes and extract cytosolic proteins selectively in a single step. We demonstrate that this approach allows monitoring the translocation of the transcription factor NF-κB from the cytosol to the nucleus without the need of subcellular fractionation.

KEYWORDS: Microfluidic, Electroporation, Subcellular, Extraction

INTRODUCTION

The 23,000 human protein-coding genes give rise to a far larger number of functional proteins due to alternative splicing and post-translational modification. To further add to the complexity, proteins also vary in their temporal and spatial organization. For example, kinases frequently move from one subcellular compartment to another (e.g. from the cytosol to the plasma membrane, or from the cytosol to the nucleus) as a consequence of their phosphorylation and activation [1-3]. Many transcriptional factors are translocated to the nucleus in response to extracellular stimuli where they bind to DNA and regulate gene transcription [4]. Thus, the study of cellular proteins in the context of their subcellular locations is important for understanding their cellular functions. Furthermore, studying a specific subcellular proteome is often a practical means to reduce the complexity of the eukaryotic cell proteome allowing the characterization of an entire proteome to become more feasible [5]. Focusing on proteins from a particular subcellular location ensures that proteins with low copy numbers do not get overshadowed by those of high abundance.

Subcellular fractionation is the most common method for preparing subsets of proteins from different locations [5]. Subcellular fractionation involves two steps: disruption of the cellular organization, typically by physical homogenization or chemical lysis using detergents, followed by differential centrifugation [6]. Physical homogenization methods lack reproducibility and often result in the incomplete release of proteins. Chemical lysis introduces detergents or reagents that often interfere with downstream analyses by tools such as mass spectrometry. Centrifugation can be labor-intensive and require bulky and expensive equipment especially when applied to large-volume samples. The process is also difficult to scale down for handling of samples of small volumes.

Electroporation is a simple physical method to breach the cell membrane barrier by applying a strong external electric field [7]. It is well established that electroporation generates nanoscale pores in the membrane of cells that allow intracellular molecules to be released into the surrounding solution [8,9]. However, the dependence of such release on subcellular location has only recently started to be understood and appreciated [10].

In this study, we report a simple method to disrupt the cell membranes and release selected intracellular proteins from a specific subcellular location in a single step. We use a high electric field to generate pores in the plasma membrane and to mobilize intracellular proteins into the surrounding solution. We show that such protein release under the electric field is highly dependent on the protein's subcellular localization: cytosolic proteins are much more readily released than nuclear proteins. We demonstrate using this approach to track the translocation of the transcriptional factor NF-κB from the cytosol to the nucleus over time without subcellular fractionation.

EXPERIMENTAL

Microfluidic devices were fabricated based on polydimethylsiloxane (PDMS) using a standard soft lithography method as described in our previous work [10].

Syk- and Lyn-deficient (Syk/Lyn-deficient) chicken DT40 B cells were described previously [2]. Harvested cells were centrifuged at 260 × g for 5 min and resuspended in an electroporation buffer (8 mM Na2HPO4, 2 mM KH2PO4, and 250 mM sucrose, pH = 7.2) at 1.5 × 107 cells/ml for electroporation.

For stimulations, CHO/GFP-NFκBp65 cells (Panomics) were incubated in serum free medium for 4 h and then placed in complete Hams F12K media supplemented with IL-1 β (Cell Science) at 40 ng/ml for indicated periods. Cells were resuspended in the electroporation buffer at a concentration of 1.5×10^7 cells/ml before electroporation.

The inlet of the microfluidic channels was connected to a syringe pump (PHD infusion pump, Harvard Apparatus) to establish steady flow rates for the electroporation. Different electric fields were established across the channel by applying various voltages. The solution from the outlet of the channel (~40 μl) was transferred to a microcentrifuge tube. After centrifugation, supernatant was collected for analysis while the pellet was washed twice before further processing.

Protein samples (the supernatant or the pellets) were separated by standard SDS-PAGE and analyzed by Western blotting. For DT40 cells, anti-p38 and anti-Sp1 (Santa Cruz Biotechnology) were used to detect p38 and Sp1, respectively. For CHO/GFP-NFκBp65 cells, anti-p65 (Santa Cruz Biotechnology) was used. The intensity of each band was quantified using ImageJ software.

RESULTS AND DISCUSSION

Fig. 1 shows the flow-through electroporation device used in this study. The cell sample flowed through a microfluidic channel with alternating wide and narrow sections while a constant DC voltage was established across the channel. As we demonstrated previously [11], the local field intensity is inversely proportional to the width of the section. Thus, electroporation occurs exclusively in the narrow sections due to the significantly higher field intensity there.

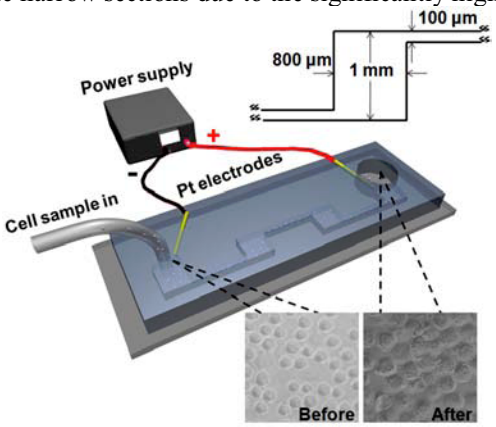

Figure 1: The layout of the electroporation device used for the selective release of intracellular proteins. The geometry of the wide sections is shown in the inset image. Each narrow section is 2.8 mm long and the channel has a depth of 60 µm. The inset images show that cells are mostly in one piece after electroporation.

In order to observe release of intracellular proteins from specific subcellular locations, we used p38 as a cytosolic protein marker and the transcription factor Sp1 as a nuclear protein marker [3]. DT40 B cells were flowed through the electroporation device to generate separate supernatant and pellet fractions as described before. The supernatant contained the intracellular proteins released into the solution by electroporation and the pellet contained the cellular "remains". We examined each fraction using SDS-PAGE and Western blotting analysis and the percentage of each protein (p38 or Sp1) present in the supernatant was calculated under specific electroporation conditions. As shown in Fig. 2, with an electroporation duration (the total residence time in the narrow sections) of 100 (Fig. 2a) or 50 ms (Fig. 2b), the release of both proteins into the solution increased with the electroporation field intensity (in the narrow sections). p38 was substantially more susceptible to electroporative extraction than Sp1 due to the difference in subcellular location. The data in Figure 2 show that flow-through electroporation provides a significant differentiation in terms of its extraction of cytosolic and nuclear proteins. For example, with field duration of 50 ms and field intensity of 400 V/cm, we were able to release 18% of the cytosolic p38 without extracting the nuclear Sp1. Alternatively, with a field duration of 100 ms and field intensities>600 V/cm, nearly all cytosolic p38 was released into the supernatant and only a small percentage (25%) of the nuclear Sp1 was released.

We are able to track intracellular protein translocation using our approach without the need for subcellular fractionation. Protein translocation refers to the change in the subcellular localization without alteration in its overall expression level. NF-κB is a family of dimeric transcription factors that regulates cellular stress responses, cell division, apoptosis, and inflammation [4, 12]. Signals from extracellular stimuli (e.g. TNFα, IL-1, LPS and DNA-damaging agents etc.) induce NF-κB to translocate from the cytoplasm to the nucleus via phosphorylation and degradation of its cytoplasmic inhibitor IκB. Such process was previously studied in microfluidic devices using fluorescence imaging when the protein of interest was tagged with a fluorescent protein marker [13, 14]. This translocation process has also been routinely studied by the combination of subcellular fractionation and Western blotting. Using our flow-through electroporation technique, we found that in general cells stimulated by IL-1β (that induced NF-κB translocation to the nucleus) retained more NF-κB after electroporation (data not shown), as expected. The difference in the electroporative release between the stimulated and unstimulated populations became very significant at field intensities 800 and 1000 V/cm (data not shown). We then used 800 V/cm and 50 ms for the electroporation and based on Figure 2b most cytosolic fraction (e.g. ~89% for p38) would be released into the supernatant together with a small percentage (e.g.~21% for Sp1) of nuclear proteins. In this case, the intracellular molecules extracted into the supernatant closely resemble the protein composition in the cytoplasm and the pellet fraction is very similar to the nuclear composition. As shown in Figure 3, by analyzing the supernatant and pellet fractions, we clearly observed the progress of NF-κB translocation from the cytosol to the nucleus over time. This confirms that our approach provides the differential extraction required by subcellular protein analysis.

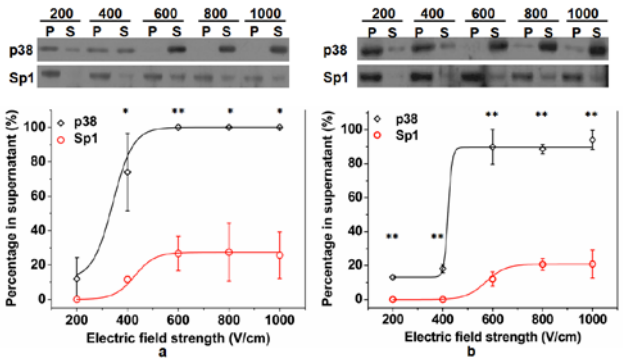

Figure 2: Intracellular protein release under different electroporation conditions. The levels of p38 and Sp1 in supernatant (S) and pellet (P) fractions from DT40 B cells electroporated at different field strengths (200, 400, 600, 800, 1000 V/cm) for 100 (a) or 50 (b) ms was analyzed by Western blotting (upper panels). The percentage of p38 and Sp1 in the supernatant fraction (calculated based on three trials of Western blot analysis) at different field strengths for electroporation of 100 (a) and 50 (b) ms is shown in the lower panels. The difference between the two data points is statistically significant with P values less than 0.05 (*) and 0.01 (**).

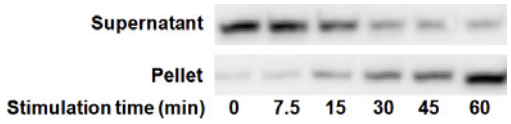

Figure 3: Tracking of NF- κB translocation from the cytosol to the nucleus over time. The intracellular proteins were extracted by flow-through electroporation and the supernatant and pellet fractions were analyzed by Western blotting after stimulation of CHO/GFP-NFκBp65 cells by IL-1β for different periods of time. We used 800 V/cm and 50 ms for the electroporation.

CONCLUSION

It needs to be noted that centrifugation, although used in the procedure to separate the supernatant and the pellet, is not an essential requirement for our method. Filtration that removes the cellular remains can achieve similar results. Depending on the studies, the supernatant, which has very similar protein composition to that of cytosolic proteins, can be used alone for analysis if a simpler procedure is desired. Furthermore, assays other than Western blotting can be coupled with the protein extraction. We envision that this technique will provide a simple and general solution to sample preparations involved in subcellular biochemistry and spatial proteomics studies.

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