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One-Dimensional Protein Analysis of an HT29 Human Colon Adenocarcinoma Cell

Zheru Zhang, Sergey Krylov, Edgar A. Arriaga,† Robert Polakowski, and Norman J. Dovichi*

Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

A single HT29 human colon adenocarcinoma cell was introduced into a fused-silica capillary and lysed, and the protein content was fluorescently labeled with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde. The labeled proteins were separated by capillary electrophoresis in a submicellar buffer and detected by laser-induced fluorescence in a postcolumn sheath-flow cuvette. Several dozen components were resolved. A number of experiments were done to verify that these components were proteins. Most components of the single-cell electropherogram had the same mobility as components present in the 30−100 kDa fraction of a protein extract prepared from the cell culture. One component was identified as a ∼100 kDa protein by co-injecting the sample with purified protein obtained from an SDS−PAGE gel. Protein expression varied significantly between cells, but the average expression was consistent with that observed from a protein extract prepared from 10^6 cells.

We have coined the term *chemical cytometry* to refer to the chemical analysis of single cells.[1] Unlike flow cytometry, which relies on light scatter and several fluorescence spectral channels to monitor a few components within a cell, chemical cytometry relies on miniaturized separation techniques and ultrasensitive detection methods to characterize cells. Edstrom reported the first chemical cytometry analysis in 1953. He used fine silk fibers to electrophoretically separate the RNA contained within a single cell.[2] Maholi and Niewisch used a fine agarose fiber in 1965 to perform gel electrophoresis separation of hemoglobin in single erythrocytes.[3] Jorgenson performed the first chemical cytometry experiment using modern analytical methods in 1987.[4,5] A single cell was lysed and its contents were fluorescently labeled in a miniature reaction vial. The reaction products were then injected into a capillary chromatography column for analysis. Ewing greatly simplified chemical cytometry by sampling a portion of the cell with the separation capillary and using electrophoresis for the analysis of the cellular contents.[6−8] Yeung demonstrated the first direct injection of an intact cell into a capillary and subsequent lysis before electrophoretic analysis.[9]

In this paper, we generate a one-dimensional protein electropherogram generated from a single cell from a human cancer cell line. A typical eukaryotic somatic cell has a 10-μm diameter, 500-fL volume, and 500-pg mass. Assuming the cell is 10% protein by weight and an average protein has a molecular weight of 25 000, the cell will contain ∼2 fmol of protein. About 10 000 proteins are expressed in each cell, with an average of 200 zmol (1 zmol = 10^{-21} mol = 600 copies) per protein. High-sensitivity protein analysis is required for single-cell protein analysis.

MATERIALS AND METHODS

Capillary Electrophoresis/Laser-Induced Fluorescence Detection. Capillary electrophoresis was performed with a locally constructed instrument.[10−12] Separation was performed in a 40-cm-long, 19-μm-i.d., and 144-μm-o.d. fused-silica capillary. The separation buffer was 50 mM phosphate, 11 mM sodium pentasulfate (SPS), at pH 6.8. Detection was performed with a locally constructed ultrasensitive laser-induced fluorescence detector, based on a sheath-flow cuvette. The cuvette had a 200-μm-square flow chamber and 1-mm-thick windows. The flow chamber was held in a stainless steel fixture, which was held at ground potential during electrophoresis. A 12-mW argon ion laser beam was used for excitation at 488 nm. The beam was focused to a ∼15-μm-diameter spot ∼50 μm downstream from the capillary exit. Fluorescence was collected at right angles with a 60×, 0.7 NA microscope objective, filtered with a 630DF30 band-pass filter from Omega Optical, imaged onto an iris to block stray light, and detected with a Hamamatsu R1477 photomultiplier tube operated at 1000 V. The sample was held in a safety-interlock equipped housing.

Cell Culture. The HT29 cell line (human colon adenocarcinoma) was grown to 80% confluence in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and 40 μg/mL gentamycin at 37 °C in 5% CO_2 atmosphere.

Protein Extract. Roughly 10^6 HT29 cells were washed five times with phosphate-buffered saline (PBS) and resuspended in

† Present address: Department of Chemistry, University of Minnesota, Minneapolis MN 55455-0431.

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100 μL of water. The cells were sonicated for 20 min at 4 °C. The suspension was centrifuged at 2500 rpm (600*g*) for 10 min. A 3-μL aliquot of the supernatant was mixed with 3 μL of water and 2 μL of 25 mM NaCN.

Protein Analysis from Cell Extract. A 250-pL plug of the protein extract containing 2.5 mM NaCN was hydrodynamically injected at 11 kPa pressure for 3 s onto the electrophoresis column. A 10 mM aqueous solution of 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) was injected for 1 s. Next, the running buffer was injected electrokinetically at 50 V/cm for 5 s. The solution was incubated at 65 °C for 3 min and then separated at 16 kV.

Single-Cell Protein Analysis. A cell suspension was prepared in 2.5 mM NaCN in PBS. A drop of this suspension was placed on a microscope slide and observed through an inverted microscope. The capillary was placed over a cell using micromanipulators. An 11 kPa partial vacuum was applied to the distal end of the capillary for 1 s, drawing the cell ~0.2 mm within the capillary. A plug of 10 mM FQ solution was injected for 1 s. The capillary tip was placed in a vial containing the SPS running buffer, and the vial was placed in a 65 °C ultrasound bath for 30 s to lyse the cell. After lysis and reaction, the separation was performed at 16 kV.

Protein Purification. One-dimensional SDS−polyacrylamide gel electrophoresis was performed on the protein extract with the BioRad Mini-Protean II system (7 cm × 10 cm minigel). A 10−20% TRIS-HCl acrylamide gel of 1-mm thickness was used for the purification. The gel was stained with imidazole and zinc sulfate solution from the BioRad zinc stain kit. The major protein band at ~100 kDa was excised from the gel and purified by the use of a Millipore Ultrafree-DA centrifugal filter. The protein sample was then loaded to hydrated Pierce Slide-A-Lyzer dialysis cassettes to be dialyzed against CE running buffer that had been diluted 1:10 in water.

Safety Precautions. Potassium cyanide is highly poisonous and reacts readily with acids to form lethal HCN gas. Stock solutions should be made in a basic buffer; neutralization of waste containing KCN should be made by addition of 1% NaOH (50 μL/g of cyanide) solution followed by slow addition of bleach (70 μL/g of cyanide).

RESULTS AND DISCUSSION

We use the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde to fluorescently label proteins. This reagent is nonfluorescent until it reacts with a primary amine in the presence of cyanide anion. The fluorescent product is excited with a low-power argon ion laser operating at 488 nm. We have reported that FQ can be used to label proteins that are lower than 10^{-12} M in concentration.[13,14]

We have reported that the use of submicellar concentrations of SDS in the separation buffer eliminates multiple labeling artifacts in FQ-labeled proteins.[13,14] In this work, we have replaced SDS with sodium pentasulfonate as a buffer additive. Figure 1 presents the separation of the protein extract prepared from roughly 10^6 HT29 human colon adenocarcinoma cells. SPS, like SDS, collapses the multiple-labeling envelope to a single, sharp peak. More importantly, there are many more components resolved in this separation than were observed with the SDS buffer. SPS appears to have a much lower affinity for neutral amino acid residues and appears to primarily interact with the ϵ-amine lysine residues. The use of this surfactant at low concentrations does not produce a constant charge-to-size ratio for proteins but instead results in a relatively large separation window.

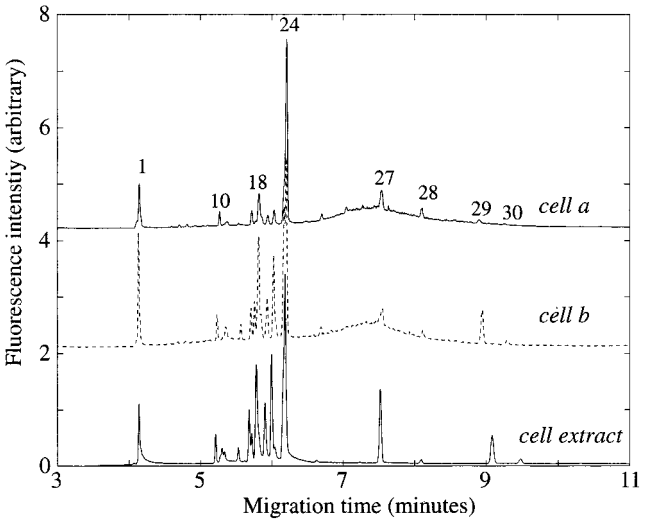

Figure 1. Free-solution electrophoresis protein analysis from HT29 cells. The cell extract was generated from roughly 10^6 cells. Individual HT29 cells were used to generate the single-cell curves.

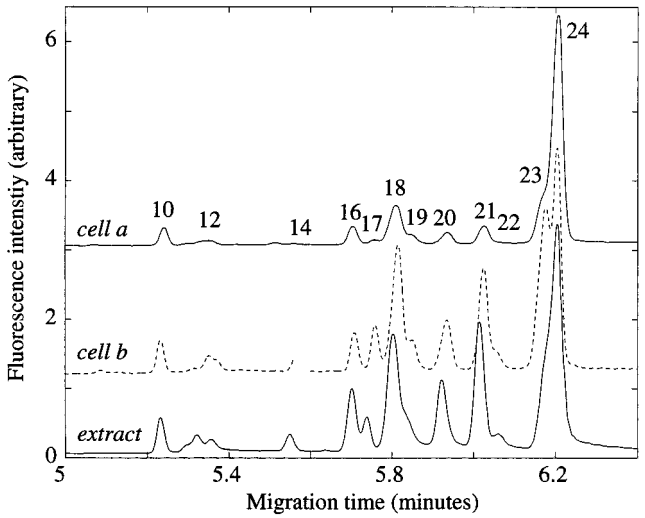

Figure 2. Detail of the single-cell protein electropherograms.

Single-Cell Protein Electropherogram. Figure 1 also presents electropherograms generated from two single cells. Each cell was introduced into the capillary using a simple vacuum system and lysed. The liberated proteins were fluorescently labeled by reaction with FQ, separated by capillary electrophoresis, and detected by laser-induced fluorescence. The protein electropherogram from a single cell was similar, but not identical, to that generated by the cell extract. At least 30 components were resolved; only the major components are labeled in Figure 1.

Figures 2 and 3 compare the middle portion of the protein electropherogram for the two cells. In all cases, plate counts are quite high, approaching 400 000. There was a noticeable variation in protein expression between the two cells. For example, peaks

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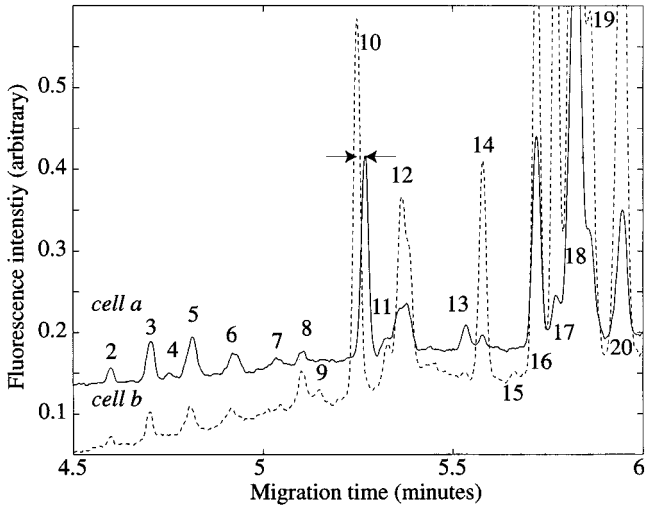

Figure 3. Detail of the single-cell protein electropherograms.

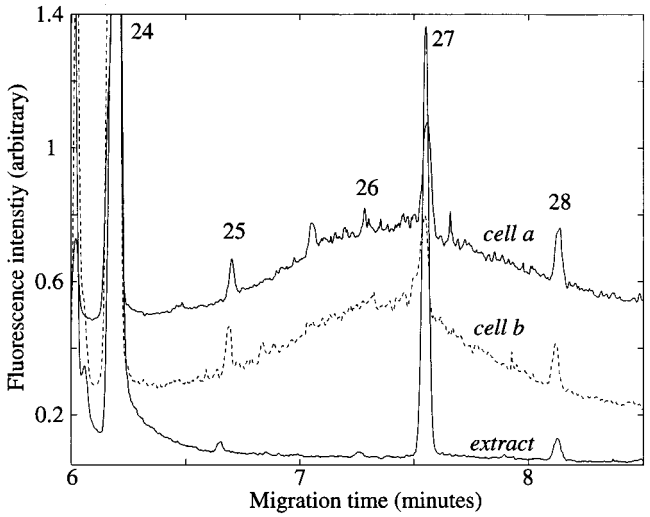

Figure 4. Detail of the single-cell protein electropherograms.

14 and 17 were barely detected and peaks 12, 19, and 22 were underexpressed in cell a. Peaks 23 and 24 were resolved into two roughly equal components in cell b, whereas peak 23 appeared as a low shoulder on peak 24 in cell a. The cell extract electropherogram was a rough average of the two single-cell electropherograms.

Peak 10, shown in detail in Figure 3, shifted to a slightly longer migration time for cell a (solid curve). This shift could be due to the disappearance of component 10 in cell b and the appearance of another component in cell a, where the latter component serendipitously has a migration time similar to that of peak 10. However, a more intriguing interpretation of the data is that peak 10 is generated by a protein and that the mobility shift represents a posttranslational modification of that protein. For example, the mobility shift could have been due to the addition of a single phosphate group to the protein in cell a, producing a slightly longer migration time.

There was a set of small peaks in the 4.5−5-min range. Most of these components were present in both cells. The minor components 9 and 15 were two of the few components that were present in cell b but not cell a.

There was a broad peak at 7−8 min in the single-cell protein electropherograms that was not observed in the cell extract electropherogram, Figure 4. We believe that this broad peak is an envelope generated by hydrophobic proteins that were lost during the preparation of the cell extract. In the single-cell experiment, the cell was lysed within the capillary and hydrophobic proteins were solubilized by the SPS in the separation buffer. The broad peak is poorly resolved because the components interact with the capillary wall. The cell extract preparation did not use a surfactant; most hydrophobic proteins were lost on the glassware used to prepare the extract and the broad peak was not observed in the cell extract electropherogram.

Some components interact strongly with the capillary wall. These components generate a large fluorescent peak when the capillary is flushed with a sodium hydroxide solution after a single-cell assay.

There was a significant variation in protein expression from cell to cell. We analyzed 5 cells and compared the protein expression for 10 of the major components. The results are summarized in Table 1. Most components had a large range in expression level, which reflects the complex biology of this cell line. These cells are likely found in different stages of the cell cycle, leading to differences in the expression of cell cycle-dependent proteins. The cells also have experienced different levels of confluence; those cells growing in close proximity to their neighbors will presumably express different levels of structural proteins. Although there is a wide variation in protein expression between cells, the average expression level is consistent with the expression level obtained from cell extracts at the 95% confidence level.

Table 1. Relative Protein Expression for Extract and Single-Cell Analysis[a]

cell extract		single-cell data[b]
peak no.	protein expression	protein expression
1	0.088	0.125 ± 0.047
10	0.041	0.039 ± 0.005
12	0.022	0.024 ± 0.009
16	0.022	0.015 ± 0.007
17	0.075	0.051 ± 0.013
18	0.139	0.123 ± 0.043
20	0.085	0.077 ± 0.031
21	0.154	0.081 ± 0.042
24	0.268	0.359 ± 0.17
27	0.106	0.106 ± 0.091

[a] Total protein expression is determined by summing the peak heights from the major components. Relative protein expression is determined by dividing the peak height by the total protein expression. Data from five single-cell electropherograms were used. The relative precision in protein expression from a cell extract was ~5%. [b] $n = 5$; mean ± standard deviation.

Flow cytometry data inevitably show a similar variability in protein expression. As a few examples, the variation in protein expression observed in our chemical cytometry data is similar to that observed for total protein content of Chinese hamster cells[15] and HeLa cells,[16] for enzymatic activity in Chinese hamster cells,[17,18] for formyl peptide binding to neutrophils,[19] and for surface

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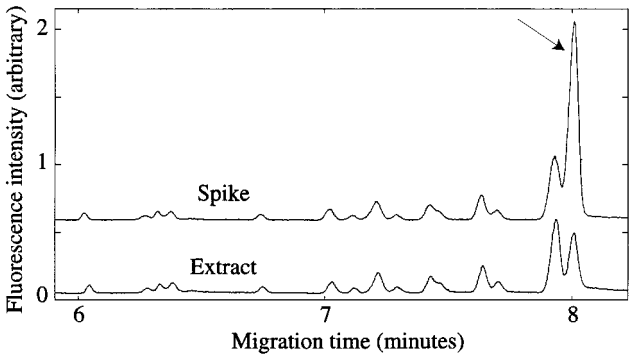

Figure 5. Identification of component 24 as a ~100 kDa protein. The entire HT29 cell extract was separated by SDS−PAGE. The gel was treated with a zinc stain, and the main component was excised from the gel, purified, and used to spike the cell extract. The bottom electropherogram was obtained from the cell extract, and the top electropherogram was obtained from the spiked cell extract. The spiked component is marked with the arrow and comigrated with component 24.

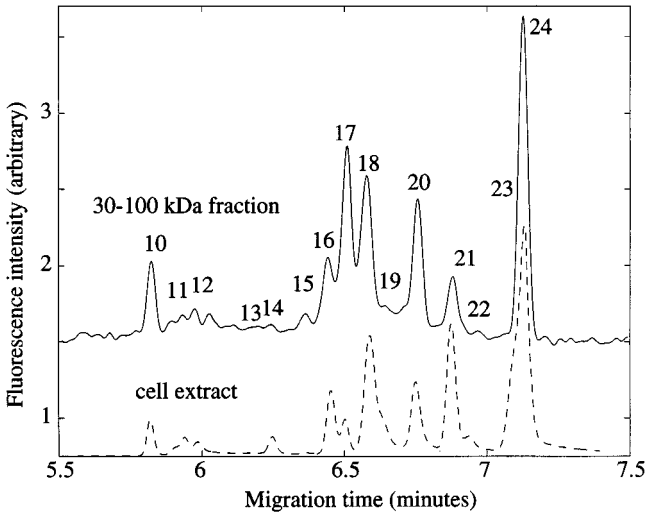

Figure 6. Verification that most components are high molecular weight proteins. The cell extract of Figures 2−5 was passed through a series of ultrafilters, and the 30 000−100 000 fraction was isolated. A slightly longer capillary was used to generate this electropherogram compared to earlier experiments. The time axis of the Figure 3 data was scaled to match the migration time of the fractionated sample; this electropherogram was added to the figure to compare the fractionated and entire cell extract electropherograms.

immunoglobulings of murine memory B cells.[20] Similar variability was observed for hemoglobin within single erythrocytes as determined by chemical cytometry.[21,22]

Protein Identification. We are in the process of identifying the components detected by the single-cell protein analysis. To test whether the peaks were due to proteins or amino acids, we mixed the protein extract with amino acid standards to generate a 10^{-7} M solution and repeated the experiment. None of the amino acids generated detectable peaks in the cell extract signal. This result is not surprising. Under our experimental conditions, FQ reacts much more readily with the ϵ-amine of lysine than with the α-amine of amino acids. The reaction discriminates against the labeling of biogenic amines and instead favors the labeling of proteins. This result is in contrast with the results obtained by Jorgenson, who used a fluorogenic reagent that appears to react more efficiently with small amines contained within single neurons.[4]

To characterize the proteins responsible for these peaks, a cell extract was separated using classic SDS−PAGE and detected by zinc ion staining. The major component had an apparent molecular weight of 100 000. This protein was isolated from the gel and was used to spike the cell extract (Figure 5). This spiked component comigrated with peak 24 in the free-solution electropherogram.

To verify that the other components were not small biogenic amines, we passed the protein extract through a series of ultrafilters to collect the fraction with molecular weight between 30 000 and 100 000. This sample was then fluorescently labeled and analyzed by capillary electrophoresis (Figure 6). This experiment used a slightly longer capillary than was used in the earlier experiments. Once scaled by an appropriate factor, the electropherogram is quite similar to that observed in the cell extract of Figure 2. Components 19 and 23 were underrepresented and components 15 and 17 were over-represented in the fractionated sample. The other components were present in proportions roughly similar to that observed in the cell extract. Most of the components in the single-cell electropherogram are generated by proteins with molecular weights ranging from 30 000 to 100 000.

CONCLUSIONS

Analytical chemists, interested in protein analysis from single cells, have used mass spectrometry to identify peptides in single giant snail neurons and hemoglobin in single erythrocytes.[24−29] However, the sensitivity of mass spectrometry is not yet sufficient to study proteins present in typical somatic cells.[29] There have been several reports of the use of capillary-based separations and fluorescence detection for the analysis of proteins from single cells;[3,22−23] these analyses were only able to detect a few major components within the cell. There have also been a number of reports of the analysis of amino acids and biogenic amines from single cells.[5,8,30−31]

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This paper presents the first one-dimensional protein electropherogram generated from a single somatic cell. The separation resolves roughly 10 major and 20 minor proteins, most with molecular weights between 30 000 and 100 000. There was also a large, broad peak that undoubtedly consists of a large number of presumably hydrophobic proteins.

There was a large variation in protein expression between cells, which was similar to the variation in protein expression observed by classic flow cytometry. The average expression level was consistent with the protein expression observed in a cell extract prepared from 10^6 cells.

The peak capacity of our one-dimensional protein electropherogram is similar to that observed for one-dimensional IEF or one-dimensional SDS−PAGE. Many more components are resolved in a two-dimensional IEF/SDS−PAGE separation. Clearly, a second separation method must be combined with submicellar electrophoresis to match the peak capacity produced by IEF/SDS−PAGE.[32]

Although the one-dimensional electrophoresis technique produces lower peak capacity than conventional two-dimensional electrophoresis, the analysis is performed on a single cell, which represents roughly a millionfold improvement in the amount of sample compared with conventional two-dimensional electrophoresis.[33] This chemical cytometry method reveals the distribution in protein expression between cells, which is hidden by classic protein analyses performed on cell extracts.

Clearly, this technology is at an early stage. It will be necessary to identify proteins that generate these peaks, to correlate protein expression with cell cycle, to generate two-dimensional maps, and to monitor the effect of therapeutic agents on protein expression. However, the single-cell protein electropherogram provides an outstanding tool to study the chemical behavior of individual cells.

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