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Asymmetry between Sister Cells in a Cancer Cell Line Revealed by Chemical Cytometry

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We introduce instrumentation and methodology for two-channel chemical cytometry of sister cells—two cells born from division of the same mother cell. The method is based on capillary electrophoresis with laser-induced fluorescence detection and allows simultaneously probing multiple intracellular components in sister cells. To test the new technology, we compared the expression patterns of green fluorescent protein (GFP) between the sisters in cultured cancer cells stably transfected with a GFP-expressing construct. We found that all sister cells had detectable asymmetry in the GFP expression patterns with a confidence level of higher than 95%. To our best knowledge, this is the first reported observation of asymmetric patterns of protein expression in sister cells in a cancer cell line. The proposed technology can reliably detect minor differences in chemical contents between sister cells, which makes it a potentially indispensable tool in studying the molecular mechanisms of developmental processes. It will be especially valuable in quantitative studies of cells with complex proliferation kinetics (e.g., stem cells).

Asymmetry in the development of sister cells (two cells born from division of the same mother cell) is crucial for embryogenesis and tissue regeneration.[1−2] Asymmetric development of sister cells is governed by the differences in protein expression between them. Until now, the only tool available to probe protein expression in sister cells was fluorescence microscopy combined with in situ immunostaining.[3] Fluorescence microscopy relies solely on spectral resolution; therefore, the number of different protein species analyzed simultaneously cannot exceed the number of optical channels available (typically less than 5). Chemical cytometry refers to the use of high-sensitivity analytical tools to characterize chemical contents in single cells; these tools include mass spectrometry, electrochemistry, and capillary separation methods. The overview of the technique can be found in a recent review article by Dovichi and Hu.[4] Chemical cytometry can provide much more detailed information on protein expression in single cells than fluorescence microscopy.[5] Technological limitations, however, so far precluded its use for the analyses of sister cells. The first limitation is associated with difficulties of reliably obtaining sister cells. The second limitation originates from a single channel used in the state-of-the-art chemical cytometers. Single-channel instruments cannot analyze two sister cells concurrently, while consecutive analyses can introduce waiting period-associated biases. In this work, we built instrumentation and developed technology for obtaining individual sister cells and their analyses by chemical cytometry based on capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection. To test the new technology, we compared expression profiles of green fluorescent protein (GFP) between the sisters in cultured cancer cells. To our best knowledge, sisters of cultured cancer cells were not reported to exhibit detectable asymmetry in biochemistry or morphology. On the other hand, tumors are characterized by significant genomic instability, which can lead to sister cancer cells having different mutations. All this makes cancer cells a challenging but interesting model for studying protein expression in sister cells. Our chemical cytometry analysis revealed that all sister cells in the 4T1 cancer cell line had detectable asymmetry in GFP expression profiles. To our best knowledge, this is the first report of detectable asymmetry in sister cells in a cancer cell line. The results prove that two-channel chemical cytometry can reliably detect minute differences in protein expression between sister cells. This makes the new technology a potentially indispensable tool in studying the molecular mechanisms of developmental processes.

EXPERIMENTAL SECTION

Reagents. Phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotics mixture, and trypsin were purchased from Invitrogen Life Technologies (Invitrogen, Burlington, ON). All other reagents were obtained from Sigma-Aldrich (Oakville, ON).

Two-Channel Chemical Cytometer. A two-channel CE-LIF-based chemical cytometer was built using a one-channel instrument as a prototype.[6] Every channel employed an individual 60-cm-long capillary (Polymicro, Phoenix, AZ) with 20-μm i.d. and 150-μm o.d. Injection ends of the two capillaries were mounted in a vertical position on an Olympus IX-71 inverted microscope (Carsen Group, Markham, ON) by means of a three-dimensional micromanipulator (Scitomix, Concord, ON) and a multipurpose

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capillary holder (Scitomix). The distance between the capillary centers at the injection ends was 0.6 mm. For CE separation, the capillary tips were immersed into a single vial with a run buffer. A single electrode and a single CZE1000R power supply (Spellmann, Hauppauge, NY) were used to drive electrophoresis. The detection ends of the capillaries were fixed inside of the sheath-flow cuvettes. Every cuvette had an individual hydraulics system to facilitate separate injection of cells into two capillaries. Light from a single Ar ion laser (Melles Griot, Nepean, ON) was split into two beams to excite fluorescence in both channels.

Obtaining Individual Sister Cells. Two lines of cultured 4T1 cells (murine mammary gland tumor cells) were used in this work: the parental line and the line with GFP stably expressed under the cytomegalovirus promoter. The cells were grown to 80−90% confluence in DMEM, supplemented with 10% heat-inactivated FBS, 50 units/mL penicillin, and 50 units/mL streptomycin at 37 °C in 5% CO_2 atmosphere. Cell suspension was prepared and single cells were transferred into individual wells on a 96-well plate by means of a capillary pipet. Individual cells were incubated for 24 h under the normal culturing conditions to allow them to divide. The sister cells were washed from the media and resuspended in PBS.

Cell Injection and CE Separation. A drop of PBS, which contained two sister cells, was placed on a microscope slide. The two cells were injected into two capillaries one after another within 1−2 min using the general procedure described elsewhere.[6] The cells were lysed inside the capillaries by SDS surfactant present in the run buffer. After cell lysis, a high voltage of +20 kV was applied to the injection ends of the capillaries (detection ends were grounded). The run buffer was 25 mM tetraborate supplemented with 25 mM SDS at pH 9.3. The capillary was rinsed with the run buffer solution for 2 min prior to each run. At the end of each run, the capillary was rinsed with 100 mM HCl, 100 mM NaOH for 2 min followed by a rinse with deionized water for 2 min.

RESULTS AND DISCUSSION

In CE-LIF-based chemical cytometry, a single cell is injected into the capillary and lysed, and its components are separated by CE and detected with LIF.[6] Unbiased analysis of sister cells requires that the two cells be analyzed simultaneously. Conventional chemical cytometers have a single channel (1 capillary and 1 detector) and thus cannot probe two cells in parallel. We have built a two-channel (2 capillaries and 2 detectors) chemical cytometer, which can analyze two sister cells simultaneously. A single high-voltage power supply was used to drive electrophoresis in both capillaries, and a single laser was used as a source of fluorescence excitation for both detection channels. To allow for accurate comparison of sister cells, the two channels have to generate similar results for identical samples. The level of difference between the two channels was measured by simultaneous CE-LIF analysis of a liquid sample, which contained several fluorescent components, four of which had signal-to-noise ratios (S/N) of greater than 100 (Figure 1A). The relative amounts of components 1−3 (normalized to the amount of component 4) had a relative standard deviation of less than 5% and differed between the two channels by less than 3% (Figure 1B).

This experiment allows us to define the criterion of confident differences between two samples measured in two channels: the samples are different with a confidence level of 95% if the difference between relative peak intensities with S/N > 100 exceeds 15%. This criterion was used to decide on whether two sister cells had different levels of protein expression.

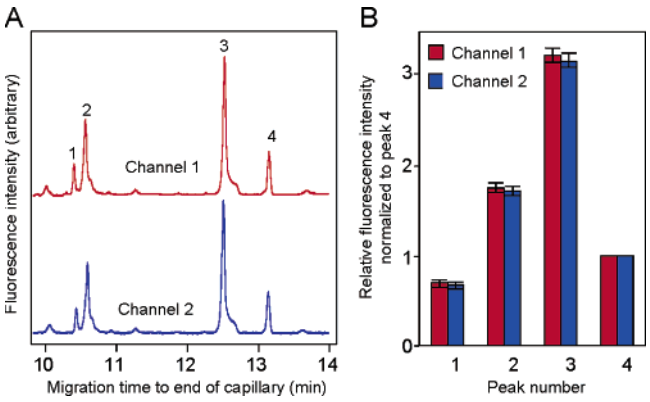

Figure 1. Response from a two-channel chemical cytometer. Panel A shows a CE-LIF separation pattern of a liquid sample, containing multiple fluorescent components, in two channels of the chemical cytometer. Panel B represents relative intensities of the four major peaks normalized to peak 4. Average values and standard deviations were obtained for 20 repetitions.

The two-channel chemical cytometer was used to compare GFP expression profiles in sisters of cultured cells. We developed the following procedure for obtaining individual sister cells and sampling them into the chemical cytometer. Single cells were seeded into individual wells of a 96-well plate and allowed to double. The sister cells were washed from the media, detached from the surface, and separated from each other. A drop of PBS, which contained the sister cells, was placed on a microscope slide. The two cells were injected into two capillaries and lysed inside the capillaries by SDS surfactant. A high voltage was then applied to the injection ends of the capillaries from a single power supply to separate the components of the two cells in parallel. This procedure guaranteed that the two cells analyzed were sisters. It also ensured that the two sisters had no other neighboring cells and thus were exposed to identical microenvironments.

Expression patterns of GFP were monitored in sister cells, in the 4T1 mammary tumor cell line, stably transfected with GFP. Control experiments with parental nontransfected 4T1 cells showed no detectable fluorescence in the GFP spectral range. For the transfected cells, we found that GFP was present in cells in multiple forms characterized by different electrophoretic mobilities (Figure 2); to our best knowledge, this is the first report on multiple GFP forms. The form with the highest amount was identified as “ordinary” GFP; this was confirmed by comparing its migration time (~13.7 min) with that of purified recombinant GFP. The nature of other GFP forms is unknown; however, all of them contain a completely translated and properly folded GFP since the maturation of the GFP fluorophore is a posttranslational process.[7] The total amount of the minor forms was less than 10% of the ordinary GFP. Significant differences in electrophoretic mobilities suggest that they can be molecular complexes of GFP with other proteins or posttranslational modifications of GFP. It is also possible that these are products of enzymatic or nonenzymatic GFP degradation at the stages at which the structure of

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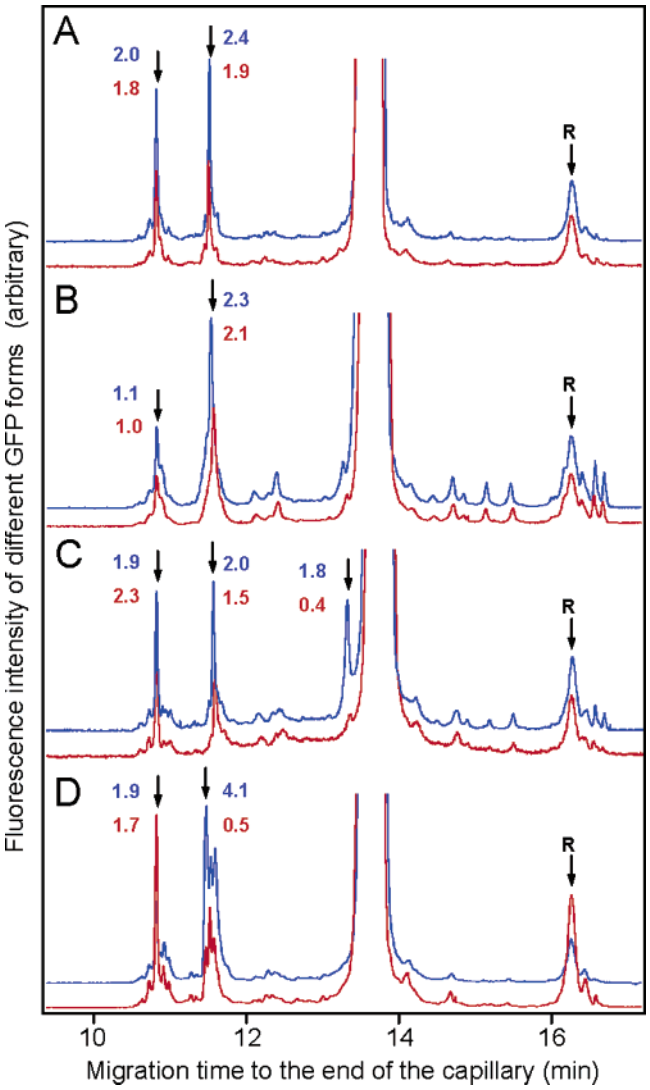

Figure 2. Differences in GFP expression patterns between sister cells in the 4T1 cancer cell line stably transfected with a GFP-expressing construct. Panels A–D show pairs of cells with varying levels of differences between the sisters. Numbers in the graph indicate relative intensities of adjacent peaks (marked with an arrow) of corresponding colors. The intensities were normalized to those of the reference peak marked with a letter R. The curves are offset along the vertical axis for clarity for viewing.

the GFP fluorophore is still unaffected. In this work, the term "expression pattern of GFP" refers to relative amounts of all GFP forms, except for the ordinary one, determined by CE-LIF. Thus, the expression pattern of GFP is dependent not only on the level of GFP transcription/translation but also on the activity of processes, which govern its posttranslational processing including catabolism. The asymmetry in GFP expression patterns of two sister cells is considered detectable if the difference in relative peak intensity for any GFP form with S/N > 100 exceeds 15% between the two cells (see above).

GFP expression patterns were compared in 32 pairs of sister cells; the patterns for 4 representative pairs with varying levels of differences between the sisters are shown in Figure 2. Although the patterns for sister cells resembled each other qualitatively, quantitative differences between them were always detectable. The sisters with the most alike GFP expression patterns had at least one GFP form with S/N > 100, whose relative amount differed between the sisters by at least 20% (Figure 2A,B). Almost half of the studied cells had GFP forms, which had S/N > 100 and differed by more than a factor of 2. Some pairs exhibited the difference in specific GFP forms (with S/N > 100) between the sisters as high as 5- and 8-fold (Figure 2C,D).

The observed differences cannot be accounted for by differences in the cell sizes, as we compared relative amounts of the GFP forms rather than absolute ones. In our experimental design, the two sisters were completely devoid of other neighboring cells and thus exposed to identical external signals. This allows us to suggest that asymmetric GFP expression patterns were imposed by asymmetry in one or more of the following: GFP expression, GFP posttranslational processing, GFP degradation, and inheritance of GFP between sisters. In the present study, we cannot distinguish between these mechanisms. The difference between the pairs of sisters is also remarkable. Most probably, it is associated with pairs being analyzed at different times after their division. The sisters' age can also explain a large range of variation between them. Presumably, the sisters are more alike immediately after division and the difference between them grows with their age.

To conclude, we have designed a powerful method to study the symmetry/asymmetry of sister cells. Using this method, we were able to observe, for the first time, the differences in protein expression profiles between sisters of cultured cancer cells. The nature of this asymmetries and their physiological relevance are unknown, but their reliable observation undoubtedly proves the power of the method. If fluorescently labeled affinity probes are used, the method will allow accurate quantitation of multiple proteins of interest in sister cells. The method will find multiple applications in developmental and cell biology—it will be especially valuable in quantitative studies of cells with complex proliferation kinetics (e.g. stem cells).

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