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Single-Cell Analysis by Chemical Cytometry Combined with Fluorescence Microscopy

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ABSTRACT

Chemical cytometry uses capillary microseparation with highly sensitive detection for detailed chemical analyses of single cells. Here, we studied microscopy and chemical cytometry in measurements of total fluorescence from single cells. An inverted fluorescence microscope was modified so that the fluorescence intensity of a sample could be automatically measured for different vertical positions of the sample with respect to the objective lens. The capillary of a custom-made chemical cytometer was mounted in a vertical position over the microscope stage. A diluted suspension of 4T1 (mouse mammary gland tumor) cells stably expressing green fluorescent protein (GFP) was placed on a microscope slide. A single cell was positioned in the center of the field of view of the microscope. The intensity of its total GFP fluorescence was measured with the microscope detector first. The cell was then analyzed in the chemical cytometer as follows. The cell was injected into a capillary and lysed to form a homogeneous cellular lysate. The lysate was driven through the capillary by pressure and its GFP fluorescence was quantified at the output of the capillary with a laser-induced fluorescence (LIF) detector. We demonstrated for the first time that in microscopy, the maximum fluorescence signal, as well as maximum signal to noise ratio, could be obtained when the cell was in one of two extremely out-of-focus positions. We proved that in chemical cytometry, the intensity of fluorescence had no memory of cell geometry and depended solely on the amount of GFP. This feature of

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chemical cytometry allowed us to use this technique as a reference method in the study and optimization of quantitative fluorescence microscopy. Using chemical cytometry as a reference method and 4T1 cells as a biological model, we proved that microscopy can be employed for reliable measurements of total fluorescence intensity from single cells in a heterogeneous population of non-synchronized cells. This finding opens the opportunity for a wide use of tandem microscopy/chemical cytometry in analytical cytology.

Key Words: Chemical cytometry; Fluorescence microscopy.

INTRODUCTION

Cytometry methods have become indispensable tools of modern cell biology. Image cytometry allows for the study of subcellular localization of target molecules,[1] while flow cytometry serves as an ideal method for the screening and sorting of relatively large populations of single cells.[2,3] Based on advanced spectral techniques and a comprehensive set of molecular probes and fluorescently labeled antibodies, image and flow cytometry allow for the analysis of several molecular targets at the single-cell level. The number of targets, which can be analyzed simultaneously, is limited by spectral resolution and typically does not exceed five. A larger number of target molecules can be monitored with chemical cytometry. The term chemical cytometry has been recently introduced to describe the application of microseparation techniques for the study of single cells.[4,5] Commonly, in a chemical cytometry experiment, a single cell is injected into a separation capillary, lysed, and chemical species of interest are separated by either electrophoresis or chromatography and detected using either laser-induced fluorescence (LIF) or electrochemistry.[6–8] Different approaches have been suggested to facilitate cell sampling for chemical cytometry.[5,9–12] Chemical cytometry has been used for the analysis of proteins,[13–15] nucleic acids,[15,16] and metabolic activities,[17–20] at the single-cell level. Moreover, the efforts are being made to use the technique for probing the contents of individual organelles.[21] With its ability of detailed chemical analysis of individual cells, chemical cytometry promises to be a powerful addition to classical image cytometry (microscopy) and flow cytometry.

Microscopy is naturally combined with chemical cytometry since cell injection into the capillary is typically performed under an inverted microscope. The same microscope, however, can be used to gather information about the biological status of an intact cell, prior to the sampling for chemical cytometry. Thus, using a tandem of microscopy and chemical cytometry, one can investigate the correlation between the biological and chemical information obtained from a single cell. Such correlations are crucial for studying the molecular mechanisms of biological processes at the single-cell level. A tandem of fluorescence microscopy and chemical cytometry has been recently demonstrated to be a powerful tool for correlating the cell cycle with the cellular metabolism.[4] In this and many other applications, the spatial distribution of fluorescence material inside the cell is not of interest. Instead, the accurate quantitation of total fluorescence from a single cell and its correlation with the biological status of the cell is desired. This correlation can be studied with a tandem of fluorescence microscopy and chemical cytometry only if both of the two techniques prove to be quantitative. Epifluorescence microscopy has been used for quantitative measurements of fluorescence

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from single cells since the 1960s.[22,23] However, due to the absence of a reliable reference method some quantitative aspects have not been addressed. As for chemical cytometry, it is a relatively new technique with its quantitative performance still being largely unknown. In this work, we studied epifluorescence microscopy and chemical cytometry in their application to the quantitation of total fluorescence from single cells.

EXPERIMENTAL

Chemicals and Solutions

All chemicals were bought from Sigma-Aldrich (Oakville, ON) and used without further purification. The components of cell growth media and trypsin were purchased from Gibco Lifescience (Grand Island, NY). Fused silica capillaries (20 μm ID, 150 μm OD) were supplied by Polymicro Technologies (Phoenix, AR). All aqueous solutions were prepared with MilliQ-quality water and filtered through a 0.22 μm filter (Millipore, Nepean, ON). Fluorescent beads (10 μm, $\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm) were purchased from Molecular Probes (Eugene, OR).

Quantitative Fluorescence Microscopy

An Olympus IX70 inverted microscope (Carsen Group Inc., Markham, ON) was used in this study. The microscope had a standard fluorescence setup with a mercury burner and a set of filters for green fluorescence (model FX115-2: exciter 450–490 nm, emitter 510–700 nm; Omega Optical Inc., Brattleboro, VT). The microscope was custom-modified to facilitate the specifics of our experiments. An electronically-controlled excitation-light shutter was used to open the excitation channel only during measurements to prevent unnecessary photobleaching of the fluorophore. The focusing mechanics was motorized with a stepping motor to allow for electronically-controlled movement of the objective lens along the vertical axis. The shutter and the stepping motor were operating synchronously. An R1477 photomultiplier tube (PMT; Hamamatsu Corp., Bridgewater, NY) was mounted on a side optical port of the microscope. The photocathode of the PMT was biased at 1200 V. The signal from the PMT was digitized with a PCI-6035E data acquisition board (National Instruments Corp., Austin, TX) and processed using a PC computer.

To measure the fluorescence intensity from a single cell, 50 μL of a diluted cell suspension (approximately 10^3 cells/mL) was placed on a microscope slide and the cells were allowed to settle. A single cell was chosen, so that there were no other cells in the vicinity (an area with a radius at least twice the radius of the filed of view) to ensure that the fluorescence intensity was measured only from that particular cell. The cell was placed in the best focus and positioned in the center of the field of view of the microscope under the bright-field observation. The microscope was then switched to the fluorescence mode to measure the cell's fluorescence intensity as a function of its vertical position.

Similar procedures were performed to measure the fluorescence intensity from samples of different cell density. In combined fluorescence microscopy/chemical

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cytometry, the cell was injected into the capillary and analyzed with chemical cytometry immediately after fluorescence microscopy measurements.

Fluorescence images of a fluorescent bead were photographed with an Olympus C3040 digital camera mounted on the microscope ocular tube. To ensure that the image was not optically contaminated by fluorescence from neighboring beads, a single bead was sampled on a microscope slide.

Chemical Cytometry

Chemical cytometry instrumentation and methodology have been described in detail elsewhere.[5] Here we outline some essentials. The run buffer was 50 mM TES, 50 mM SDS at pH 7.3; SDS was necessary for cell lysis. The analysis was carried out in a 40-cm long fused silica capillary (20 μm ID, 150 μm OD). Approximately 3 mm of the outer polyimide coating was stripped from both ends of the capillary after burning it in a gas flame. The injection end of the capillary was held in a vertical position in a custom-made light-transparent capillary holder. The capillary holder also held a platinum electrode in close proximity to the capillary to facilitate electrophoresis (not used in this study). Another feature of the capillary holder was a gas channel bored through its body with an opening near the capillary. The channel was connected to a cylinder of pressurized helium gas and facilitated a pressure-driven flow through the capillary. In this work, only pressure-driven flow was used. The capillary holder was mounted under the same Olympus IX70 microscope that was used for quantitative fluorescence microscopy. The detection end of the capillary was mounted in a sheath-flow detection cell of the following dimensions: 200 × 200 μm inner bore, 2 × 2 × 20 mm outer size (Hellma Canada Ltd., Concord, ON). The detection cell, in turn, was mounted in a custom made stainless-steel holder. The sheath flow around the capillary facilitated hydrodynamic focusing of the analyte stream coming out of the capillary similarly to focusing in flow cytometry. Fluorescence excitation light from an Ar-ion laser (488 nm, 12 mW; Melles Griot, Ottawa, ON) was focused with a 4× microscope lens (Melles Griot, Ottawa, ON) on the analyte stream at a distance of approximately 20 μm from the capillary orifice. Fluorescence of the analyte was collected at a right angle to the excitation beam with a 60×, 0.7 NA microscope objective (Universe Kogaku Inc., Oyster Bay, NY). To eliminate scattered light, fluorescence was filtered with a bandpass filter centered at 520 nm with a transmission window of 40 nm (Omega Optical Inc., Brattleboro, VT). The fluorescence was detected with a PMT (model R1477; Hamamatsu Corp., Bridgewater, NY) biased at 900 V. The signal from the PMT was digitized using a data acquisition board (model PCI-6035E; National Instruments Corp., Austin, TX) and processed with a PC computer.

Fifty microliters of a cell suspension ($\sim 10^3$ cells/mL) was placed on a microscope slide coated with polyvinyl alcohol and the cells were allowed to settle. The capillary was placed in the center of the field of view of the microscope in a vertical position, using a custom-made 3-dimensional micromanipulator mounted on the microscope stand. The tip of the capillary was immersed in the cell suspension at a distance of approximately 50 μm from the surface of the microscope slide. The capillary was pre-filled with the run buffer containing SDS to facilitate cell lysis. A single cell chosen for the analysis was superimposed with the inner bore of the capillary by moving the microscope stage. The capillary was lowered to approximately 10–15 μm from the microscope slide and the cell

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was injected into the capillary by suction induced with a pulse of negative pressure (1 sec × 9 kPa) applied to the opposite end of the capillary. The cell was drawn into the capillary along with a ~0.3 mm plug of phosphate buffered saline (PBS) followed by a ~0.3 mm plug of the SDS-containing run buffer. The SDS diffused from the run buffer towards the cell and lysed it within a 1-min incubation period. The tip of the capillary was then placed into a vial containing the run buffer. The run buffer was then pressurized by the helium gas at 480 kPa to move the cellular lysate through the capillary. The fluorophore's fluorescence was detected while exiting the capillary. The capillary was rinsed with the run buffer solution for 2 min before each run. At the end of each run, the capillary was rinsed with 100 mM NaOH for 2 min, followed by a rinse with deionazed water for 2 min.

Cell Preparation

The 4T1 cell line stably transfected with green fluorescent protein (GFP) was kindly donated by Dr. Chuan Li (Duke University, Durham, NC). The cells were cultured in Dulbecco's Modified Eagle's MEM (DMEM) supplemented with 10% fetal bovine serum and 1% Antibiotic-Antimycotic at 37°C in 5% CO_2 atmosphere. Immediately before using them, the cells were treated with 0.25% (m/v) trypsin at 37°C for 10 min to promote their detachment from the dish. The cells were then centrifuged at 250× *g* for 5 min to form a pellet. The pellet was washed three times with PBS and resuspended in PBS to avoid background fluorescence from the media. The working density of cells in the suspension was approximately 10^3 cells/mL.

RESULTS AND DISCUSSION

Quantitative Fluorescence Microscopy

We aimed at measuring the total fluorescence from single cells. To avoid background fluorescence from neighboring cells a diluted cell suspension was used to ensure that only a single cell was in the field of view of the microscope. The cell was placed in the center of the field of view and in the best focus under the bright-field observation. The microscope was then switched to the fluorescence mode and the fluorescence intensity was measured for different vertical displacements of the cell from the "in focus" position.

The fluorescence intensity was found to be a complex function of the vertical position (*z*-position) of the cell (Fig. 1, line 1). The fluorescence intensity profile (*z*-profile) had two maxima with a minimum between them. These two maxima were almost 200 μm away from the focus position. This distance was markedly larger than finite depth-of-focus for objective lenses used. The focus position corresponded to neither the maximum nor the minimum, but it was close to the latter. Qualitatively similar *z*-profiles were obtained with objective lenses of different magnifications (60×, 40×, and 20×) and hence with different numerical apertures and depths of focus.

To check whether the observed *z*-profile was a unique feature of single-cell measurements, we increased the number of cells in the field of view of the microscope. Figure 1 compares *z*-profiles of fluorescence intensity for a single cell (line 1), a 5-cell cluster (line 2), and a cell monolayer (line 3). We found that the more cells were in the field

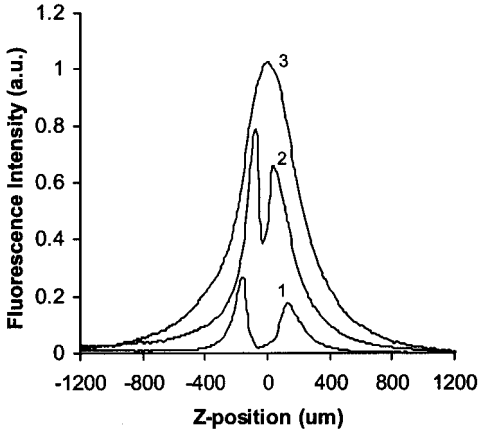

Figure 1. z-Profiles of fluorescence intensity for a single cell (line 1), 5 cells (line 2) and a cell monolayer (line 3) were compared using a 60×, NA 0.7 objective lens. Zero in the x-axis represents the in-focus position.

of view, the closer the fluorescence maxima were. Two maxima merged forming a single peak with maximum intensity corresponding to the "in focus" position when a monolayer of cells was analyzed. Thus, the complex two-maxima z-profile was characteristic for analyses of single cells or low-density cell suspensions. The explanation of the observed z-profile was out of scopes of this work. Our goal was rather to assess whether the two positions corresponding to maximum fluorescence could be used for reliable quantitative analysis of total fluorescence from single cells. This issue is addressed in the last section of this paper.

Along with a finite depth of focus, the complexity of the single-cell z-profile implies that the fluorescence intensity from a single cell may depend not only on the total amount of fluorophore, but also on cell geometry (size, shape, and intracellular distribution of the fluorophore), and cell position with respect to the objective lens. Although due to the complexity of the optical system, it is very difficult to calculate this dependence, it is possible to experimentally study the extent to which the geometric factors affect the fluorescence intensity for any chosen cell type. Such experiments require that along with fluorescence microscopy, the amount of the intracellular fluorophore be quantified with an independent reference method. Below, we prove that chemical cytometry can be used as a reliable reference method to study and optimize quantitative fluorescence microscopy of single cells.

Chemical Cytometry

First, we examined the linearity of chemical cytometry with respect to the amount of fluorescent material introduced into the capillary. Identical plugs of fluorescein solutions of different concentrations were injected into the capillary and driven by pressure through the capillary. The area under the time profile of the fluorescence signal (Fig. 2, line 1) was

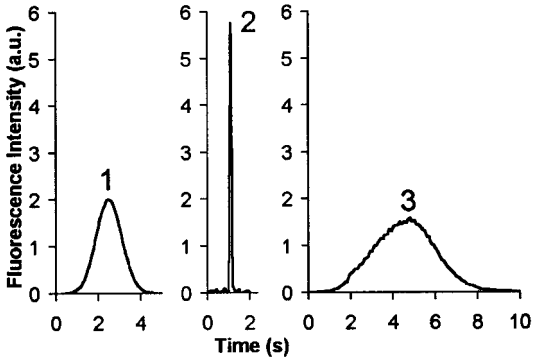

Figure 2. Pressure driven time-profiles of different analytes in chemical cytometry: a plug of fluorescein solution (line 1), an intact single 4T1-GFP cell (line 2), and a lysed 4T1-GFP cell (line 3).

found to depend linearly on the amount of injected fluorescein within the whole dynamic range of the LIF detector.

Then, we considered the requirements for fluorescent signal depending solely on the amount of intracellular fluorophore and not on the cell geometry. In chemical cytometry, in general, a cell is injected into a capillary and lysed. The cellular lysate is moved through the capillary and the fluorescence intensity of the lysate is quantified at the output of the capillary. The fluorescence intensity will depend only on the amount of intracellular fluorophores and will not depend on cell geometry if: (i) the fluorophore is completely solubilized in the run buffer so that it passes the detector in a uniform stream of a homogeneous solution and (ii) the geometry of the detector does not change from run to run. We used the LIF detector of a fixed geometry; thus the second requirement was satisfied automatically. To examine whether or not the first requirement was satisfied, we analyzed the time-profiles of the fluorescence signal generated by intact cells and a cellular lysate. To completely prevent cell lysis in the capillary and ensure that an intact cell passed the detector, we used the run buffer that did not contain the lysing agent, SDS. The intact cells generated very sharp fluorescence peaks, which saturated the PMT (Fig. 2, line 2). Then, we used the SDS-containing run buffer that lysed the cell. Due to the diffusion of GFP along the capillary, the fluorescent peak was much broader for the lysed cell (Fig. 2, line 3) than that for the intact one (Fig. 2, line 2). The peak corresponding to the lysate was smooth indicating that no non-solubilized fluorescent particles passed the detector (if such particles had been present then we would have observed sharp spikes). It is worthwhile to mention that comparing the peak areas for lines 1 and 3 allowed us to estimate the absolute amount of GFP in a single cell, providing a practical way of determining absolute amounts of fluorophores in single cells.

Due to complete cell lysis, the signal in chemical cytometry has no memory of cell size, shape, and intracellular distribution of fluorophores. Thus, chemical cytometry is a reliable technique for quantitative analysis of fluorescence from single cells. This finding allowed us to use chemical cytometry as a reference method in the study and optimization of quantitative fluorescence microscopy.

Chemical Cytometry as a Reference Method for Fluorescence Microscopy

Whether or not the geometry of the cell and its $x-y-z$ position affected the fluorescence signal in microscopy was assessed by correlating fluorescence intensities measured with microscopy and chemical cytometry. Thirty single GFP expressing 4T1 cells were analyzed by tandem fluorescence microscopy/chemical cytometry. The cells were chosen randomly from a non-synchronized cell population. As a result the amounts of their intracellular GFP varied significantly. We found that the z-profiles were similar with respect to the z-positions of two extrema for all cells.

Fluorescence intensities of single cells measured with microscopy and chemical cytometry were then compared. Four distinctive points in the z-profiles were used for this comparison: the three extrema and the "in-focus" position (Fig. 3). Fluorescence intensities measured with microscopy in all 4 z-positions showed liner dependence on that measured with chemical cytometry. The correlation coefficients for all four z-positions were identical and close to 1 (0.985). In other words, for any of the four z-positions, the total fluorescence intensity of single cells measured with the fluorescence microscope was proportional to the total amount of the fluorophore in the cells.

The linearity between the intensity signals suggests that the natural slight variation in cell size in the non-synchronized population of 4T1 cells was not to the extent that could

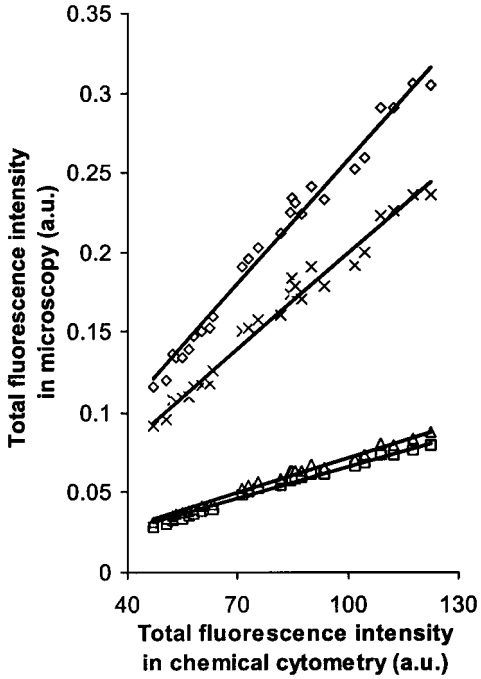

Figure 3. Correlation between chemical cytometry and fluorescence microscopy for four z positions used in microscopy: the "in-focus" position (△), the z-position corresponding to minimum of fluorescence (□), and the z-positions corresponding to the two fluorescence maxima (◇ and ×).

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cause non-linearity between intensity signals. In other words, although in theory the fluorescence signal in microscopy could depend on cell geometry, we found that the cell size did not affect the fluorescence signal considerably, due to relative size homogeneity. This observation is particularly important since it proves that fluorescence microscopy can be a reliable tool for single cell fluorescence quantitation for non-synchronized cell populations.

The fluorescence signals and signal-to-noise ratios were about 3-fold higher in the *z*-positions corresponding to the maxima. Moreover, the *z*-positions corresponding to the maxima could be easily determined in an operator-independent manner, while the "in-focus" position is subjective and prone to uncertainties associated with human judgment of the image quality. Therefore, it seems to be quite practical to use the maximum fluorescence position for fluorescence quantitation from single cells. In our optimized tandem microscopy/chemical cytometry, the cell is first placed in focus to study the cell's morphology using bright-field observation. Then, the microscope is switched to the fluorescence mode and the cell to objective distance changes automatically to generate the *z*-profile. The fluorescence intensity can be then found as the maximum signal. The *z*-scanning stops when the cell is returned to focus. The microscope is switched back to the bright-filed mode to facilitate simultaneous observation of the cell and the capillary during cell sampling into the capillary for chemical cytometry.

CONCLUSIONS

To conclude, we proved that chemical cytometry allows the measurements of total fluorescence intensities from individual cells in a manner that is dependent only on the amount of intracellular fluorophore and independent on the cell size, cell shape, and the intracellular distribution of fluorophores. With chemical cytometry as a reference method, we demonstrated that epifluorescence microscopy can also be a reliable tool in the measurements of total fluorescence intensity from single cells. These findings open the opportunity for a wide use of tandem microscopy/chemical cytometry in analytical cytology.

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