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Cycling into Future: Mass Cytometry for the Cell-Cycle Analysis

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Progress in the development of instrumentation and new methodologies is a driving force for advancement in biology and medicine. By offering a possibility to assess cell heterogeneity and identify individual cells or cell subpopulation having different properties, combined with multiparametric analysis, the cytometric methodologies are of particular value. Compared with the early means to interrogate individual cells by microspectrophotometry and autoradiography developed in 50s and 60s of the past century, the introduction and advances of flow cytometry during the last four decades revolutionized the way of cell analysis and contributed to the immense growth of our knowledge in many disciplines of cell and molecular biology. It had also found important clinical applications advancing diagnosis and treatment of many diseases (1). Analytical capabilities of exploring individual cells were further enriched by the development of imaging cytometry instrumentation, such as provided by laser scanning cytometry and imaging flow cytometry. Progressive improvements in flow and imaging cytometry led to unprecedented expansion of our knowledge of regulatory mechanisms in normal and diseased cells. Particularly, fruitful were applications of these methodologies in revealing mechanisms of cell proliferation as related to a multitude of molecules, signaling pathways and events controlling the cell-cycle progression (1).

We are witnessing now a dawn of new technology, the mass cytometry, which represents a quantum leap in expansion of analytical capabilities probing individual cells (2–6). Cell labeling, including by immunocytochemical means, is done with stable isotopes of rare earth metals that ordinarily are not present in the cell, which are being detected and measured by mass spectrometry. This technology evades limitations of flow cytometry such as the need for compensation of the emission spectra and offers a possibility for multiparametric analysis unparalleled by any other methodology. Thirty-four parameters have been concurrently measured in individual cells of human bone marrow assessed for differentiation status, including analysis of their differentiation pathways with the use of phosphospecific antibodies (Abs) (3). Potentially, at least 45 parameters can be measured simultaneously in single cells with rates approaching 1,000 cells per second (4).

In the article presented in this issue (page 552), Behbehani et al. describe adaptation of mass cytometry to analysis of the cell cycle. In this study, the key components of the cell-cycle progression machinery cyclin A and cyclin B1 were identified with the respective Abs tagged with the transition elements. Iododeoxyuridine was successfully used as a marker of DNA replicating cells and histone H3 phosphorylated on Ser10, detected with the transition element-tagged phosphospecific Ab, was the marker of mitotic cells. The DNA intercalating ligand containing iridium was applied to mark DNA and assess its content (6), whereas immunocytochemical detection of phosphorylated on Ser807 and Ser811 retinoblastoma

protein (Rb) epitope was helpful to distinguish cycling from noncycling (G_0) cells. Using several cell lines and mitogenically stimulated lymphocytes, the authors compared cell-cycle distributions obtained by the mass cytometry with the data provided by the classical methods of cell-cycle analysis by flow cytometry. The mass cytometry approach was validated by yielding cell-cycle distributions of the measured cell populations in very close agreement to those obtained by classical fluorescence cytometry techniques.

Although this study is the first attempt to utilize mass spectroscopy for comprehensive analysis of the cell cycle, the authors succeeded to identify all phases of the cell cycle, including a distinction of G_0 from G_1 and mitotic from G_2 cells. As the status of phosphorylation of pRb is a well-recognized landmark of a transition between the off- and on-cell-cycle state (7), therefore, it was an excellent choice for discriminating between the cell in- and off-the cell cycle. This approach has already been used by flow cytometry; indeed, the ratiometric analysis of phosphorylated to nonphosphorylated pRb has been found to be a good parameter distinguishing noncycling from cycling cells (8).

There is a long list of markers, including RNA content (9), susceptibility of DNA in situ to denaturation (10) or expression of protein detected by Ki-67 Ab (11), discriminating between cycling and off-the cell-cycle cells. It should be noted that although, in principle, each of these parameters can identify cells that are progressing through the cell cycle from the cells that are off the cycle they differ in terms of distinction of the “depth” of cell quiescence and thus they are not fully equivalent (10). Having status of pRb phosphorylation [p-Rb(S807/S811)] as a biomarker, Behbehani et al. identified three populations of cells: a pRb(S807/811)¹ which was defined as cycling population, a pRb(S807/811)^{mid} defined as G_0 cells, and the unlabelled pRb(S807/811), which appears to represent predominantly dead (apoptotic) cells. In our prior studies by measuring cellular RNA content (9) or DNA in situ susceptibility to denaturation (10), we were able to distinguish three populations of cells with DNA content of $G_{1/0}$ cells, which were defined as G_{1Q} , G_{1A} , and G_{1B} . G_{1Q} cells having minimal RNA content and DNA most susceptible to denaturation, reporting the greatest degree of chromatin condensation, were the cells in deep quiescence. An example of G_{1Q} cells are peripheral blood lymphocytes which upon mitogenic stimulation have to progress through G_{1A} and G_{1B} , and are required at least 24 h, to enter S phase. The G_{1A} cells, seen in exponentially growing cultures (and not present among nonstimulated peripheral blood lymphocytes) had medium RNA content and moderate susceptibility of DNA to denaturation. They were postmitotic cells in the growth phase synthesizing RNA and growing in size until their RNA content reached a certain threshold level. The G_{1B} cells had RNA content above this threshold and were stochastically entering S phase (10). There was no evidence of the presence of G_{1Q} cells in exponentially growing cultures of all cell lines that have been studied (10). In their studies, Behbehani et al. observed the presence of pRb(S807/811)^{mid} cells in cultures of all four cell lines. Considering the above, it is evident that the G_0 cells defined by the pRb phosphorylation status [pRb(S807/811)^{mid} cells] include both the G_{1Q} and the G_{1A} cell subpopulations recognized by the RNA content or DNA susceptibility to denaturation. It is likely that the noncycling cells identified by the lack of expression of Ki-67 antigen (11,12) may also not be equivalent to G_{1Q} or pRb(S807/811)^{mid} cells. It is of importance, therefore, that while identifying noncycling cells to define the marker that is being used to discriminate this subcompartment of the cell cycle.

The obvious extension of these findings was an application of this methodology to analysis of the cell cycle in various subsets of hematopoietic cells in healthy human bone marrow. The authors were able to identify 34 cell subpopulations at different stages of maturation by immunophenotyping and measure and record the cell-cycle phase distributions, including the distinction between G_0 and G_1 cells, for each of these subsets. This analysis and the

obtained results are of fundamental importance, providing the stepping stone and the setting to compare changes in cell-cycle distribution in different hematological diseases that may be seen in further studies. Application of this methodology to detect and characterize cell-cycle abnormalities in hematological proliferative diseases, particularly in different types of leukemia, is expected to provide a wealth of information of great diagnostic and prognostic values. As cancer is a disease of the cell cycle, the information as to which hematopoietic cell subset has cell-cycle abnormality and what character of this abnormality is, may be essential in designing the personalized treatment protocol.

Despite the success in identifying all phases of the cell cycle by mass cytometry by Behbehani et al. (page 552), there is still room for improvement. Specifically, not much stoichiometry in binding of intercalating ligand containing iridium to DNA is apparent. Improved stoichiometry of the DNA marker will markedly increase resolution in identification of the cell-cycle phases. Perhaps cisplatin, the drug having affinity to bind DNA and widely used in cancer treatment, may provide better quantitative marker of DNA than the iridium intercalator. One would expect, however, that soon new intercalating or minor-groove binding-specific DNA ligands containing heavy metal atom, with the improved resolution in assessment of DNA content, will be developed to find use in mass cytometry. This improvement will be of importance in clinical application of this technology particularly to identify aneuploid cells concurrently with immunophenotyping and their cell-cycle analysis.

Taking into an account the multiparametric capabilities of mass cytometry, addition of further markers in analysis of the cell-cycle machinery is possible. Among the key interesting markers are the status of phosphorylation of tumor suppressor p53, expression of CDK inhibitors of the Cip/Kip and INK4/ARF families, activation of Chk1 and Chk2 protein kinases, expression and phosphorylation status of members of Cdc25 family proteins and expression of Bax. Combined analysis of the growth signaling pathways with these markers will immensely enhance our ability to gain insight into molecular mechanisms of cell-cycle regulation and explore aberration of these mechanisms in diseased cells. Development of mass cytometry opened a new, postfluorescence era of cytometry (4) which will further accelerate progress in biology and medicine.

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