High Content Image Cytometry

A Versatile Tool for Mapping Subnuclear Organization

The human eye is a powerful and sensitive instrument which excels in pattern recognition, by using mental templates to identify and categorize objects. Unfortunately, its exact strength may also be its weakness, for it implies a certain subjectivity to the interpretation of images. Furthermore, our distinctive power and vocabulary for describing objects in a quantitative manner are limited. Accurate visualization and characterization of cellular features thus calls for a more objective approach, which can now be provided by the fast growing discipline of high content image cytometry (HCIC).

Image Cytometry: State of the Art

With high contrast and a resolution that surpasses the human eye by a factor of 1000, fluorescence microscopy provides the suitable ground material for documenting intracellular patterns. Digital analysis of these images, in turn, enables extraction of quantitative information about the cell through detection and measurement of multiple spectrally and spatio-temporally resolved signals (i.e. high content). To achieve a reproducible and robust cytometric assay automation and integration of both acquisition and analysis are essential (fig. 1). Where automation increases acquisition speed, image dimensions and capacity (i.e. high-throughput), integration creates possibilities for feedback and real-time interaction. We have combined a fully automated microscope with in-house developed image analysis tools and data mining techniques, to establish an integrated cytometric platform that is flexible and allows performing a large variety of biological assays, focused on the biology of the cell nucleus.

The Human Cell Nucleus as Diagnostic Tool

The nucleus is considered to be as a central hub which dynamically coordinates the cell's activities in varying environmental conditions. Within the limited space of the nucleus the entire genome and many proteins are accommodated in a non-random
manner. This meticulous organization has functional relevance to the cell, assuring proper gene expression, replication and genome stability [1]. Therefore, nuclear organization may prove a valuable indicator for a variety of pathologies.

Thorough understanding of this spatio-temporal complexity requires a multi-parametric analysis, preferably on large numbers of cells. In other words, image cytometry is a must, because it allows relating subcellular protein expression patterns to cellular condition and, by extension, to human health.

A typical example of how to gauge cellular health is by scoring the amount of DNA damage. DNA damage can lead to loss of genetic information, genome rearrangements or cell death, and therefore give rise to pathologies. Exposure of cells to a variety of genotoxic agents, such as chemicals or ionizing radiation, results in DNA double stranded breaks. Upon recognition of a double stranded break the histone protein H2AX is rapidly phosphorylated leading to the formation of visually discernable γ-H2AX repair foci in the nucleus. Quantification of these foci by manual counting is highly subjective and error-prone, especially when few spots are present. Furthermore, the amount of cells that can be evaluated is low. The high variability between individual cell responses makes acquisition of large datasets preferable. Clearly, in this case, manual evaluation is no longer an option. Therefore, we have devised an automated assay to score γ-H2AX foci formation in human fibroblasts after exposure to X-irradiation (fig. 2a, 2,3). On average ~500 cells were screened per condition - from acquisition to analysis - in less than 10 min. We consistently retrieved a clear dose and time dependence and could distinguish between conditions in quantitative terms using a variety of descriptive parameters such as spot number, spot size and spot occupancy. Thus, our approach of digital titration clears the road for biodosimetry at the level of the single cell.

Bearing this in mind, we applied the same setup to the analysis of human fibroblast
cell cultures which were sent to space (3). Why space? Beyond the protective atmosphere of the Earth, astronauts are exposed to a broad spectrum of ionized atomic nuclei (cosmic rays), including high-energy (HZE) particles which deposit their energy along well-defined tracks to produce (DNA-) damage. To allow accurate biodosimetry, it is not only of interest to measure DNA-damage response in cells that were directly hit by HZE-particles, but also in neighboring cells to evaluate the contribution of cellular communication (the bystander effect). Using automated mosaic microscopy and track detectors to colocalize HZE-impacts with DNA damage (γ-H2AX response), we were able to measure the local response in cell cultures at the site of impact as well as in its direct surroundings (fig. 2b). This illustrates the unique advantage of HCIC in allowing cellular processes to be studied within their (pseudo-) natural context.

**Connecting the Dots, a Machine-Based Approach**

So far, we have discussed the analysis of known or expected end-points. However, the true strength of HCIC lies in the multitude of information which, at first sight, may not seem relevant, but when combined or put in perspective may reveal novel insights in underlying networks or interrelationships. Unfortunately, the massive amount of data increases the risk of losing the overall picture. To alleviate this issue, we have implemented data mining and machine learning techniques, which made it possible to recognize and pinpoint specific nuclear phenotypes.

We illustrate this with the analysis of transfected cells (fig. 3). Transfection of cells with fusion constructs allows studying functional as well as dysfunctional (cfr. disease-related) proteins within their natural context. However, transfections usually result in a variety of phenotypical outcomes with varying expression levels between cells. Both scoring of transient transfection efficiency and monitoring of the quality of stable transfectants, could benefit from objective and automated analysis. Reliable classification of the different phenotypes can be obtained by using their cytometric fingerprint, which, in turn, is generated by exhaustive extraction of parameters describing nuclear shape, signal intensity and specific protein localization. The classification procedure is first optimized by making use of a training set of images of transfected nuclei with known phenotypes. This way, individual nuclei can be classified according to their specific expression pattern and/or level. Evidently, this approach can be generalized to almost any cytometric challenge.

**Conclusion**

We and others have applied HCIC to the quantification of DNA damage [2,3] or
characterization and classification of protein localization patterns [2,4]. These examples underline the importance of systematic analysis of subnuclear features in large image data sets. For, accurate classification of phenotypes at the level of the single cell may be the starting point in the development of diagnostic and/or prognostic tools for human health monitoring. The current trend of increasing scale and dimensions can only contribute to the power and robustness of these assays. In this context, the combination of different channels allows multiplexing the analysis. Gaining even more relevance is the time dimension. Analysis of living cells is constantly improved by innovations that allow prolonged imaging and reduce photodamage [5,6]. Moreover, automation and integration are blurring the boundary with high content applications, thereby heralding the era of cytomics at the in fluxo level [7]. Clearly, there is a strong growth potential for HCIC. Given that living systems are dynamic and dynamics are an essential component of organization, it is safe to say the future for high content cytometry is bright and in 6D.

References

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