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Going into Lengths and Widths, and Depths—Microscopic Cytomics Quantifying Cell Function and Cell Communication

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ALTHOUGH microscopy has always been an integral part of cytometry and especially of flow cytometry, this basis has long been forgotten. The first quantitative measurements, performed by Casperson et al. (1) on a microscope in the early 1950's, ultimately led to the development of a commercially available slide-based cytometer in the 1990's by Kamentsky et al. (2). The rapid distribution of this instrument among scientists, cell biologists, and some clinicians, has made this fundamental basis of cytometry more visible again: "Cytometry is coming home." The various characteristic capacities of slide-based versus flow cytometry have been outlined in part previously. In recent times, two major issues attract our attention:

1. The specimen on the slide can be evaluated repeatedly and the information obtained can be assigned to the cells on a single-cell basis, i.e. "individually." This opens the door to an n-parametric analysis as has been outlined by Mittag et al. 2006 (3).

2. The cells can be kept within their natural environment i.e. the tissue and their connection with the neighborhood can be analyzed.

Schubert (4) was the first to exploit this feature of the slide-based design and presented the concept of toponomics: similarly as it is important not only to what amount a protein is expressed but also its exact location in the cell. Also of substantial impact is the identification of what kind of neighboring cells surround a given cell of interest.

Together with these theoretical concepts, delicate hardware support has been developed. Whereas Clatch et al. (5) have originally described an immunophenotyping method using a simple glass slide modified by adhesive strips and later applications took plain glass for cell immobilization, recently several authors have presented elaborate solid supports for repeated single cell analysis. A novel approach to the immense complexity of the cellular immune system is developed by Hennig et al. (6). Iterative restaining on a slide-based platform is successfully used to measure an almost unlimited set of markers of cell differentiation and cell function in living cells as was previously outlined theoretically (7). This assay takes commercially available slides and yields very detailed data on single cells with instrumentation that is rather inexpensive. It needs minimal sample size and shows excellent correlation with flow cytometry.

Tajiri et al. (8) use a specifically produced microwell chip for combining a functional assay (cytokine secretion) with the analysis of intracellular proteins and surface markers. The microwell design offers the possibility to cob the cells in a liquid environment allowing short time culture and time-lapse analysis. If analyses had included more than one fluorochrome, even more detailed data could have been obtained. However, depending on the condition of the cells the illumination needed for fluorophore bleaching could also have a directly modulating effect on cell function and could therefore interfere with functional analyses.

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Tracking the same cells by time-lapse analysis is also an issue concerning the matter of neurite growth and branching. First steps for machine-based tracing of individual neurites were achieved by Meijering et al. 2004 (9) by developing NeuronJ. However, obtaining quantitative data requires time-consuming post-acquisition data manipulation. To improve this, Popko et al. (10) introduce the automated analysis software XL-Calculations allowing batch analysis to yield quantitative data on the growth of neurites at a level of detail that has not been previously reported. Yu et al. (11) chose another way to obtain data on neurite outgrowth and introduce NeuronCyto. Using dynamic watershed lines for keeping topological dependence, their software yields quantitative data on neurite growth on a single-cell basis. It can be applied even in dense cell cultures using cellular images. Both approaches, XL-Calculations and NeuronCyto, determine length, branching complexity, and number of neurites. The authors hope for applications in neuroregeneration.

A further step deeper into the analysis of cell-interaction in natural conditions is modeled by Händel et al. (12). They apply their model system to mouse colon tissue and investigate the direct neighboring of regulatory T-cell characterized by Foxp3-expression and proliferating cells detected by ki67. Although it does not give a definitive diagnosis and is not intended to do so, this approach for quantitative tissue analysis or tissomics (13) could yield therapeutically relevant data that so far are available only on a subjective basis if at all. Another "close-to-clinical" application is presented by Goodale et al. (14) who characterize tumor cell dissemination applying a combination of flow and slide-based cytometry. Both platforms yield comparative data in a preclinical setting. Their quantitative analysis of tumor spread might represent the kinetics relevant in patients as well.

Quantitative analysis in cytometry however is not limited to static expression of markers or production of stimulated cytokines but can also be applied to movement as shown by Kamgoué et al. (15). The ability to analyze the shortening of single sacromeres in cardiomyocytes impressively outlines the capacities of slide-based assays as they can follow contraction waves traveling along the cell. It allows quantifying the cardiomyocyte contraction without the need of fluorescent calcium probes and yields an excellent spatiotemporal resolution.

High-throughput single-cell based analysis is applied by Matula et al. (16) to quantify viral infection in an automated manner. They make use of a novel gradient-based thresholding scheme for cell nucleus segmentation which turned out to be of particular use in densely packed cell clusters. The capacity of this image-based approach is underlined by the ability to quantify viral replication in transfected cells on siRNA cell arrays. This is an excellent model for industry scale high-throughput assays and makes genome-wide screening possible.

The 3D-organization of a cell is of particular relevance in cell biology, and its quantitative or even automated analysis is a demanding task. Pinidiyaarachchi et al. (17) developed a method for detecting and localizing fluorescent signals generated by molecule complexes and were able to accurately measure the distance of these complexes to the nuclear membrane after stimulation. It transfers the concept of toponomics into live-cell analysis and allows one to spot ligand-induced translocation "red-handed."

A true masterpiece of cell analysis is performed by Du and Wasser (18) who took the challenge of 3D imaging of *Drosophila* muscles. They developed a fully-automated algorithm for the acquisition of 3D-image stacks of single living muscles and to compensate for non-linear movement due to periodic contractions and developmental changes. It was used to show single muscles undergoing apoptosis in early stages of *Drosophila* metamorphosis.

In conclusion, this issue of Cytometry Part A collects the latest achievements in quantitative microscopic analysis of biological specimens. The articles impressively highlight the capacities of current quantitative cell-based assays. Since the parameters are analyzed in their cellular context they open the door to a cytome-based understanding of diseases, leading back to Virchow's *Cellularpathologie* (19) centered on the cell as the ultimate unit of life. Instead of analyzing a cell-shake that has lost any information about the histological and cytological topology as in standard proteomic assays (20,21) these cytomic techniques allow us to perform locational proteomics in its truest sense. This background raises the hope that these assays will make their way to clinical applications. This development gets more and more visible for the last two years; during that time several special issues of Cytometry Part A have focused on high-content cytometry and systems biology (22,23) to which this issue serves as an update.

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