Cytometry in the brain: studying differentiation to diagnostic applications in brain disease and regeneration therapy

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Abstract

During brain development, a population of uniform embryonic cells migrates and differentiates into a large number of neural phenotypes – origin of the enormous complexity of the adult nervous system. Processes of cell proliferation, differentiation and programmed death of no longer required cells, do not occur only during embryogenesis, but are also maintained during adulthood and are affected in neurodegenerative and neuropsychiatric disease states. As neurogenesis is an endogenous response to brain injury, visible as proliferation (of to this moment silent stem or progenitor cells), its further stimulation can present a treatment strategy in addition to stem cell transfer for cell regeneration therapy. Concise techniques for studying such events *in vitro* and *in vivo* permit understanding of underlying mechanisms. Detection of subtle physiological alterations in brain cell proliferation and neurogenesis can be explored, that occur during environmental stimulation, exercise and ageing. Here, we have collected achievements in the field of basic research on applications of cytometry, including automated imaging for quantification of morphological or fluorescence-based parameters in cell cultures, towards imaging of three-dimensional brain architecture together with DNA content and proliferation data. Multi-parameter and more recently *in vivo* flow cytometry procedures, have been developed for quantification of phenotypic diversity and cell processes that occur during brain

development as well as in adulthood, with importance for therapeutic approaches.

Quantifying and characterizing cells in the brain

Comprehensive analysis of functional anatomy of the brain aims at quantitative examination of components of the cortex, using multiple fluorescence colours, determination of DNA-ploidy, cell cycle phases and apoptosis, as is routinely performed on cultured cells and peripheral blood leucocytes. Components of cell networks can be visualized using fluorescence microscopy. Expression pattern of cell markers can be manually counted from randomly selected visual microscopic fields, compared to total cell numbers (counted using nuclear counterstaining with DNA dye) and cell processes analysed. However, numbers of earlier studies have questioned reproducibility of manual evaluation of microscope images by trained experts, particularly when deciding between labelled and unlabelled cells (1,2). Thus, there is a need for automated and unbiased approaches for quantitative analysis in brain sections as well as in three-dimensional brain architecture.

Fluorescence-based cytometry (flow cytometry – FCM or imaging cytometry – IC) is a convenient alternative to immunohistochemical methods for quantifying cells in a fast and reliable way. Appropriate standardization procedures and continuous quality control warrant objective evaluation and quantification of cell type distribution, antigen expression patterns and intracellular signalling networks. However, FCM-based measurements of brain tissue have in the past been extremely challenging. This partly is due to the complex and interwoven structure of neurons that may extend over very long distances in the central nervous system, and are virtually impossible to be isolated as intact cells. Analysis of neuronal cells with their morphological features is usually possible only in cell culture.

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For FCM applications, preparation of single cells or nuclei from solid tissue blocks is necessary. This can be performed using different methods, such as mechanical dissociation, grating cells from a tissue block, touch preparation, touch smears or fine-needle aspirated biopsies. Although these methods are well established for tumour material, they are not useful for brain tissue. Obvious disadvantages in treating brain samples in such a way are that cytoplasm and membranes are removed during such preparation. Obtained nuclei are often unsuitable for FCM analysis, as display of cytoplasmic proteins (e.g. cell type-specific markers or neurotransmitters) is necessary for discrimination of different cell types. Moreover, by preparing single cells, morphology of the tissue as a whole is lost, making further anatomical and pathological analysis impossible. Even so, several investigators developed FCM-based approaches to isolate, identify phenotypic marker expression, and to enrich cells or cell compartments from the brain. This includes synaptic terminals from the cerebral cortex of Alzheimer's disease (AD) patients (3), CD44 expression in mouse cerebellum (4), cell death as measure of annexin V/propidium iodide-stained ischaemic brain tissues (5) and fluorescence-activated cell sorting of neurons, astrocytes and microglia, from the nucleus accumbens (6).

Stem cells and neurogenesis in embryonic and adult nervous systems

The central nervous system develops from a small number of cells proliferating and interacting in a very particular manner to form a functional neural network with regional identities over hundreds of billions of cells. This process is initiated when neural stem cells (NSC) are induced to proliferate and migrate, resulting in differentiation of neurons and glial cells (mainly astrocytes), under the control of growth and neurotrophic factors (7). In addition, other types of neural stem cell originate from the neural crest (8), which (along with further tissue types) originate the peripheral nervous system.

Proliferation and subsequent neural differentiation of stem and progenitor cells is not restricted to the brain during development, but also occurs during adulthood, mostly associated with conditions of neuronal loss due to traumatic brain injury or neurodegenerative disease. Adult neurogenesis occurs in the subventricular zone of the forebrain and subgranular zone of the dentate gyrus within the hippocampus (9). The potential of stem cells, of neural and non-neural origin, for differentiation in the neural direction, as well as their potential in cell therapy for brain diseases, has also been extensively stud-

ied. Pericyte-like stem cells and mesenchymal stem cells from bone-marrow, adipose tissue and dental-pulp, express neuron-specific marker protein expression, upon transdifferentiation stimuli (10,11).

Adult neurogenesis is a life-long process with other tissue-specific stem cells. Activation of quiescent stem cells involves cell cycle entry from G0 to G1/S phase to increase the population that is able to develop into new neurons. For onset of differentiation, cell cycle blocking is necessary, followed by phenotype transition until the terminally differentiated state has been reached. The importance of inhibition of cyclins and cyclin-dependent kinases by members of the Kip and Ink family as major players in NSC cell cycle regulation, underlying neurogenic proliferation and differentiation, has previously been detailed in a further review of our group (12).

The potential of neural stem and progenitor cells for proliferation is affected in a number of disease conditions. Neurogenesis can be induced in experimental epilepsy and brain ischaemia, while reduction in proliferation, and survival of subventricular zone progenitor cells in a mouse model of Parkinson's disease has been observed. Yet, brains of patients suffering from Huntington's disease or Alzheimer's disease reveal enhanced proliferation of neural progenitor cells (13), indicating that the role of endogenous NSC in pathophysiology of neurodegenerative diseases needs to be further elucidated.

Cytometry-based techniques have become important for identifying stem cells, using a panel of phenotype markers for detection. Nanoparticles and novel fluorophores such as 'Brilliant Violet' reporters, much brighter than other UV-excitable dyes (14), have drastically increased numbers of epitopes measured simultaneously with increased sensitivity. These provide spectrally distinct options for FCM, fluorescence imaging, two-photon microscopy and IC.

For decades, proliferation stimulation has been assayed based on DNA measurement, detection of proliferation markers and incorporation of thymine analogues into replicating DNA. A common proliferation assay measures BrdU (5-bromo-2'-deoxyuridine, an analogue of thymidine) incorporation, which then is detected by a detectable antibody marking the S-phase cells. EdU (5-ethynyl-2'-deoxyuridine), another thymidine analogue, has advantages over BrdU, as EdU does not result in epitope destruction, permitting co-staining with antibodies, and characterizing cell phenotype and stage of differentiation. Sun *et al.* (15) have described a method that combines EdU incorporation and labelling of cell surface antigens for multi-parameter flow cytometric phenotype analysis, together with biological parameters.

Novel strategies for working with rare cell populations for example in the analysis of cell cycle status by BrdU, Ki-67, and Pyronin Y staining, have been developed (16). Moreover, recently sophisticated molecular biology strategies have focused on development of Ki67-driven GFP-marker gene expression, making detection of proliferating cells much more sensitive (17). Figure 1 shows currently used techniques for FCM- and IC-based detection of cell cycle progression and proliferation.

However, most of cited techniques are restricted to analysing *in vitro* cell cultures or cells isolated from

tissues and deprived of their natural environment. Thus, *in vivo* FCM has been developed for cell tracking in small animals, without the need to draw blood samples, and are particularly useful for detection of stem cells or cancer cells (18,19). A breakthrough lies in the work of Zhang *et al.* (20) describing an integrated optical system that combines laser scanning confocal microscopy and *in vivo* flow cytometry for simultaneous visualization and cell quantification. The system is set up specifically for non-invasive tracking of both stationary and circulating cells in adult zebrafish engineered to be optically transparent. Although this system has only been tested so far with a modified organism (transparent cells/zebrafish), it is clear that future applications of cytometry lie in the analysis of stem cell differentiation in their *in vivo* niche. It is expected that already existing techniques for *in vivo* brain imaging will be revolutionized with the introduction of wide spectra bright and specific fluorescent probes. These are used with instrumentation allowing three-dimensional localization and detection of target epitopes within the embryonic and adult brain. *In vivo* imaging systems permit detection of neural progenitor cells and follow up progress of neurogenesis and other physiological functions in the live brain. A number of nanoprobe has been prepared, evaluated and applied, in various imaging modalities including fluorescence, magnetic resonance, radionuclide, Raman and photo-acoustic imaging (reviewed in 21).

Near-infrared fluorescence nanoprobe for malignancy molecular detection have been developed for cancer imaging, providing high-sensitivity spatial resolution while being low risk to the organism, as the only radiation it emits is non-ionizing. Such a nanoprobe consisting of a specific peptide/EGF receptor, modified with various lengths of mono-discrete polyethylene glycol units and near-infrared Cy5.5 fluorescence dye has been developed and tested on target cells overexpressing EGF receptor for glioblastoma imaging in live animals (22).

Image cytometry to study the cell cycle in normal brain, disease and development

Image cytometry combines the virtues and advantages of quantitative FCM and (immuno)histochemistry. It enables multiparametric analyses to be performed, while preserving morphological characteristics, on fresh or fixed tissue sections on microscope slides. Thus, not only cell suspensions as in FCM can be investigated, but also cells in their intact structural context. In general IC is a non-confocal setup, but the microscope is used with low numerical aperture thereby assimilating a relatively high depth of field up to 5–20 μm . This allows whole cells to be analysed and amounts of their

Figure 1. Cytometry techniques for detection of cell cycle progression and proliferation. Ki-67 immunostaining labels cells during cell cycle progression [G1, S, G2 and mitosis (M)], but not cells in the quiescent G0 phase. BrdU- and EdU-incorporation assays detect cells progressing through the cell cycle in S, G2 and M phases, while in combination with DNA staining by propidium iodide, cells in G1, G2 or M phase are distinguished. Cyclin-B1 is a G2-phase specific marker.

respective constituents, such as total DNA content, to be quantified. Thus, IC is well suited for *in situ* identification, classification and analysis of cells and subcellular structures. These features render IC a powerful tool for research and diagnostic applications. It is useful for quantitative analysis of different neural cell types and cell constituents with single or multicolour staining, morphological details of brain tissue and two- and three-dimensional distribution of cells in tissue sections.

Mosch *et al.* (23,24) established a sample preparation procedure applicable for brain tissue, by cutting 30 and 120 μm frozen sections, and staining them after fixation and permeabilization. Samples were used for imaging cell distribution within the tissue and discrimination of neural subtypes, including neurofilament⁺ cells with dysregulated cell cycles, as observed by expression of cyclin B1, a G2 phase marker. Neurofilament⁺ cells could be observed without any alteration in the cell cycle (Fig. 2). The thick tissue sections were scanned at a variety levels of microscopic focus. Obtained images were organized by x-y coordination for production of a single data file with three-dimensional distribution of cells in of the brain section (Videos S1 and S2).

Use of thick sections prevents possible artefacts and subsequent errors, which may occur when using thin sections (5 or 10 μm). Such an example is provided by truncated nuclei, which appear to have lower DNA content (hence disturbing the cell cycle analysis), among other tissue damage resulting from mechanical stress. The huge amount of data occurring in three-dimensional

analysis is yet difficult to handle; however, advancement of software and hardware components will turn such approaches into standard techniques. Compared to other methods for analysis of tissue sections such as histology and microscopy, IC affords standardized and fast measurement of specimens and simultaneous quantification of multiple fluorescent signals (25). For example, the IC protocol has been optimized for more efficient DNA content quantification in a very large number (500 000) of identified neurons (23,26).

However, first applications so far, using IC for analysis of intracellular concentrations of specific protein at single cell level, were reported subsequently. Reinert *et al.* (25) used laser-scanning cytometry-based multicolour tissue analysis for identifying a subpopulation of neurons expressing a novel form of extracellular matrix, perineuronal net, and analysing ferritin H concentration in the parietal cortex of the rat brain. In the normal human brain, Fischer *et al.* (27) quantified frequency of neurons with higher than diploid content of neuronal DNA in different cortical areas, and analysed changes during ageing. Around, 11.5% neurons had a DNA content above the diploid level. Gender did not affect DNA content (mean values $\pm$ SD, men: $11.41 \pm 2.95\%$; women: $11.59 \pm 2.57\%$).

A main challenge addressed by IC was whether there was cell cycle dysregulation in brain cells of AD patients. As hypothesized by Yuri *et al.* (28), ongoing neurodegeneration and consequent disease progression involve a mechanism of ectopic cell cycle events.

Figure 2. Laser-scanning cytometry imaging of human parahippocampal gyrus. Left image: Illustration of the parahippocampal gyrus within x-y-z-position of laser-scanning cytometry software showing cyclin B1-positive and negative cells and morphological details. Marked in blue and green are cyclin-B1-negative and -positive neurons, respectively. Right image: Combination of different measurements showing three-dimensional distribution of cells in the brain section [images reproduced with authorization from Figs 1 and 2 of Mosch *et al.* (23)]. Green: neurofilament-positive, cyclin-B1 negative cells; red: neurofilament-positive, cyclin-B1-positive cells; black: unlabelled cells.

Neurons subject to neurodegeneration exhibit biomarkers of cell cycle progression and DNA replication, suggesting re-entry into the cell cycle. However, these cells are not capable of completing the cell cycle and undergoing mitosis, finally resulting in their dedifferentiation and degeneration (29). Furthermore, the generally recognized amyloid cascade theory (30), defining deposition of beta-amyloid as the keystone in initiation of AD, does not account for observations of why, for instance, transgenic mice overexpressing amyloid precursor do not develop the predicted cascade (31, reviewed in 32), suggesting further possible important mechanisms for disease development. Genomic instability (aneuploidy) in the AD brain resulting from reactivation of the cell cycle and partial or full replication, is thought to contribute to AD progression (reviewed in 33). Neurons with an aneuploid set of chromosomes are also present at low frequency in the normal brain while numbers of aneuploid neurons in AD brain tissue are highly elevated, coincident with cell death of neurons with this chromosomal aberrance.

Based on previous studies mentioned above, work of Mosch *et al.* (24) has provided evidence for the possibility that replication stress, resulting from failure of affected cells in completing mitosis, would disturb the (epi)genomic landscape in the brain and lead to DNA replication 'catastrophe' causing cell death during the S phase (replicative cell death). DNA replication stress can be a key element of a pathogenetic cascade, explaining the interplay between ectopic cell cycle events and genetic instabilities in the AD brain, and also contributing to observed aneuploidy in this disease. A connection of dysfunctional cell cycle re-entry resulting in replication stress with somatic genome variation provides an important novelty in cell cycle and molecular mechanisms of AD.

This hypothesis has been generated on the basis of IC-generated results of quantitative analysis of neurons of the entorhinal cortex in individuals with different degrees of AD progression (24). As expected, the number of neurons, as shown by anti-neurofilament immunostaining, was reduced, while at the same, the fraction of neurons expressing cyclin B proliferation marker, and their fraction re-entering the cell cycle and stagnating in early S-phase, substantially increased. From the technological aspect, this study also evaluated the quantitative ability of IC technology to measure cell cycle alterations at the single cell level. Results obtained by three independent methods, IC determination of DNA content, chromogenic *in situ* hybridization of α -satellite sequences of the centromere of chromosome 17, and real-time PCR quantification of ALU repeats (high copy number motif with low level of polymorphism), revealed high inter-method reliability. Thus, DNA content quantification by IC

performs as well as real-time PCR quantification of repetitive sequences or chromosome counting. It can measure as many as 1000–100 000 cells in a short time, thereby providing information at the individual cell level.

Interest in studying cell cycle parameters and aneuploidy is not restricted to neurodegenerative diseases. Aneuploidy, visible as gain or loss of genetic material (chromosomes), here of neurons, glia and neural progenitor cells, is thought to participate in producing neuronal complexity, intercellular diversity and evolutionary changes (34). In line with such hypothesis, formation of mosaics of intermixed aneuploid and euploid cells also is present during neural development. Recent work has defined aneuploidy of neural progenitors as a parameter for programs of neurogenesis and cell death (35). Conditions in attenuating programmed cell death, by using a combination of methods for visualization and counting of chromosomes and determination of DNA content by FCM has resulted in markedly enhanced mosaicism, observed as increased numbers of cells with numerical aneuploidy including extreme forms, such as loss or gain of five chromosomes. Here the authors concluded that there was functional non-equivalence of different karyotypes with mild aneuploidies being preserved over extreme forms, determined by programmed cell death or survival of distinct karyotypes. As an effort to detect rare karyotypes, a FCM-based imaging approach was developed to detect chromosomal abnormalities following FISH in suspension. This enables automated analysis of several log-magnitude higher numbers of cells compared to microscopy-based approaches (36). Nevertheless, modern IC techniques will allow for following such patterns in the developing animal, determining differentiation and cell death parameters and DNA content in a three-dimensional tissue complex.

Quantitative IC analysis and detection of rare cells

Quantitative determination of cell constituents or biological parameters in individual cells is possible, almost exclusively only, with quantitative IC instrumentation for measurement and (semi-) automated analysis. In many cases, quantification of the parameter of choice is a matter of manual analysis, which often lacks reproducibility, due to misjudgement of defining cell boundaries *versus* background, as explained below. Recent achievements have been gained for defining countable items and automating such procedures.

Analysis of fluorescence microscopy images of cells often requires determination of membrane edges to separate cell components in an image from the surrounding background. Segmentation accuracy varies with imaging

Table 1. Comparison between FCM and IC techniques for measuring neural cell parameters *in vitro* and *in vivo*

Aim of study/best model	Methods	Advantages	Disadvantages
DNA-ploidy/ <i>in vivo</i>	Fluorescence microscopy	Maintains cell morphology information/qualitative	Manual evaluation/poor reproducibility
DNA-ploidy/ <i>in vivo</i>	Image cytometry	Maintains morphology, fast measurement/multiple fluorescence signals/quantitative	Manual analysis lacks reproducibility
DNA-ploidy/ <i>in vitro</i>	Flow cytometry	Fast and reliable/quantitative	Impossible to isolate intact cells
Apoptosis/ <i>in vivo</i>	Fluorescence microscopy	Maintains cell morphology information/qualitative	Manual evaluation/poor reproducibility
Apoptosis/ <i>in vitro</i>	Flow cytometry	Fast and reliable/quantitative	Impossible to isolate intact cells
Cell cycle/ <i>in vivo</i> & <i>in vitro</i>	BrdU-fluorescence microscopy	Maintains cell morphology information/qualitative	Manual evaluation/poor reproducibility/denatured epitopes
Cell cycle/ <i>in vitro</i>	BrdU-flow cytometry	Fast and reliable/quantitative	Denatured epitopes
Cell cycle/ <i>in vitro</i>	EdU-flow cytometry	Fast and reliable/quantitative	Native conformation of epitopes
Cell cycle/ <i>in vivo</i>	Image cytometry	Intact whole cell fast measurement/multiple fluorescence signals/quantitative	Manual analysis lacks reproducibility
Circulating cells/ <i>in vivo</i>	<i>In vivo</i> flow cytometry	Intact organism	Modified transparent animals
Specific cell markers/ <i>in vivo</i> & <i>in vitro</i>	Fluorescence microscopy	Maintains cell morphology information/qualitative	Manual evaluation/poor reproducibility
Specific cell markers/ <i>in vitro</i>	Flow cytometry	Fast and reliable/quantitative	Impossible to isolate intact neural cells

FCM, flow cytometry; IC, imaging cytometry.

conditions that determine sharpness of cell boundaries and with geometric features of a cell. Dima *et al.* (37) presented a bivariate similarity analysis method for indicating how algorithms fail, and present an edge quality metric for prediction of image segmentation success.

Computational methods for image structure detection and quantification should be reliable as long as foreground signal exceeds the background. However, as with any image segmentation algorithm, obtained results ultimately depend on image quality.

HCA-Vision software (CSIRO Computational Informatics, North Ryde, Australia) is a fully automated image analysis solution for generating neurite traces, for segmenting the cell soma and for associating neurites with their respective soma on a per-cell basis, even when cells touch each other (38).

Yu *et al.* (39) have developed a tracing algorithm, NeuronCyto, to automatically trace neurites and measure their lengths quantitatively on a cell-by-cell basis of neuroblastoma cells, used as model for neuroregeneration. Analysis of neuritogenesis is frequently performed by Sholl analysis or by counting total number of neurites and branch tips. Manual analysis of large data sets often fails due to lack of reproducibility. Moreover, such analysis can obscure subtle differences, as data sets are not available in a form which would make application of multiple analytical tools possible. Al-Kofahi *et al.* (40) developed a program for automated tracing of neurite branching points from 3D-confocal microscopy images. They claimed the program is valuable for ensuring completeness of extracting neuronal topologies, and

for high accuracy in neurite profiling, outgrowth, and toxicology assays that require counting of branch points.

To overcome limitations of manual analysis, the 'Bonfire' program was developed to facilitate digitization of neurite morphology and subsequent Sholl analysis (41). A complete review of this has been published by Meijering (42) on development and perspectives of software tools for the study of neuronal anatomy, including neuritogenesis. One step further on the way to routine automated image analysis and reliable detection of rare cells (which could be stem or tumour cells in the brain), has been taken by Scholtens *et al.* (43). They have developed an IC detection system, CellTracks TDI, for quantifying fluorescence and measuring morphological parameters at high sensitivity and resolution, with the capability of relocating events for subsequent analysis. Extending the above-cited work, the same research group has developed a system for automated classification of events collected with CellTracks TDI system. The automated classifier, based on the Random Forest method, distinguishes between different cell types such as circulating tumour cells from leucocytes, as well as between tumour cell debris and particles not related to tumour cells. Obtained classifications were in agreement with results collected with the automated classifier or by researchers experienced in the subject.

Conclusions

In summary, quantitative tissue analysis is currently available to examine different regions and cell types in

brain tissue. These methods identify and evaluate large numbers of individual cells, cell decisions (including proliferation, differentiation and cell death), as well as morphological changes occurring along with differentiation. FCM technique can be applied to detecting expression of multiple target molecules in a large numbers of brain cells simultaneously, such as exemplified in ref. (44). However, utilization of this technique is limited to analysis of cells grown in culture and not applicable to tissues or cell populations, which need to be analysed as a network. IC techniques reveal information at the single cell level, allowing identification of marker protein expression and cell functions in tissues, and reorganizing obtained images in a three-dimensional setup. Table 1 reports advantages and disadvantages of FCM and IC in various applications. Manual IC analysis is known to be time consuming, and interpretation of data may vary between scientists performing analysis; however, efforts have been undertaken for automating and speeding it up. Overall, advancement of cytometry techniques and applications increases our understanding of cell networks and biological processes in healthy and diseased brain.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Videos S1 and S2. Videos show 3D reconstruction of a brain section (part of the parahippocampal gyrus) from a series of confocal images measured on an iCys Laser Scanning Cytometer, CompuCyte Corp., USA). Green: neurons (NeuN staining), red: cell nuclei (propidium iodide) and blue: cyclin B staining. 3D reconstruction and the video were prepared by Jens-Peer Kuska († 2009) and Ulf-Dietrich Braumann, both IZBI – Interdisciplinary Center for Bioinformatics, University Leipzig, Germany.