Assessment of DNA replication in central nervous system by Laser Scanning Cytometry

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Abstract

In neurons of patients with Alzheimer’s disease (AD) signs of cell cycle re-entry as well as polyploidy have been reported\textsuperscript{1,2}, indicating that the entire or a part of the genome of the neurons is duplicated before its death but mitosis is not initiated so that the cellular DNA content remains tetraploid. It was concluded, that this imbalance is the direct cause of the neuronal loss in AD\textsuperscript{3}.

Manual counting of polyploidal cells is possible but time consuming and possibly statistically insufficient. The aim of this study was to develop an automated method that detects the neuronal DNA content abnormalities with Laser Scanning Cytometry (LSC).

Frozen sections of formalin-fixed brain tissue of AD patients and control subjects were labelled with anti-cyclin B and anti-NeuN antibodies. Immunolabelling was performed using Cy5- and Cy2-conjugated secondary antibodies and biotin streptavidin or tyramid signal amplification. In the end sections of 20\textmu m thickness were incubated with propidium iodide (PI) (50\mu g/ml) and covered on slides. For analysis by the LSC PI was used as trigger. Cells identified as neurons by NeuN expression were analyzed for cyclin B expression. Per specimen data of at least 10,000 neurons were acquired.

In the frozen brain sections an automated quantification of the amount of nuclear DNA is possible with LSC. The DNA ploidy as well as the cell cycle distribution can be analyzed.

A high number of neurons can be scanned and the duration of measuring is shorter than a manual examination. The amount of DNA is sufficiently represented by the PI fluorescence to be able to distinguish between eu- and polyploid neurons.

Key words: Alzheimer’s disease, cell cycle, DNA-polypoidy, Laser Scanning Cytometry

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1 Introduction

Alzheimer’s disease (AD) is a devastating dementia of late life, it is associated with a region-specific accumulation of senile plaques containing deposits of Aβ-peptide, formation of neurofibrillary tangles, formed by paired helical filaments (PHF) including abnormally highly phosphorylated tau protein and extensive neuronal cell loss. Despite of partial uncovering the etiology of the disease, the cause of neuronal death remains unknown. Recent findings support a link between neurodegeneration in AD and cell cycle related events. Whereas in physiological conditions cell division in neurons is predominantly present during development of the nervous system, several laboratories have offered evidence that the attempt to re-enter the cell cycle mediates neuronal cell death in AD. This hypothesis is emphasized by the findings that cell cycle related proteins such as cyclin-dependent kinases (cdk 1, 4 and 5), cyclins D, E, B and A, cyclin-dependent kinase inhibitors p16, p15, p18, p19, p21 and p27 and also proliferation-associated proteins such as p105, Ki67 and PCNA are re-expressed in variable subsets of neurons in brains of AD-patients. The fact that mitotic events participate in AD-pathology is not only seen in the expression of cyclin B and cdk1 but also in the formation of mitosis-specific phosphoepitopes in potentially vulnerable neurons prior to neurofibrillary degeneration and the association of these phosphoepitopes with PHFs. Taken together there is evidence that cells re-enter the cell cycle. Since post-mitotic cells get stuck before finishing the cell cycle in G2, they die rather than divide. A remaining question however is whether the immunohistochemical appearance of cell cycle proteins is indicative of a true cell cycle or only the dysregulation of protein synthesis. Direct proof of DNA replication missed in the Alzheimer’s disease brain until Yang et al. (2001) performed fluorescence in situ hybridization (FISH) to examine human hippocampus in autopsy material from Alzheimer’s disease patients and controls. The authors found direct evidence for attempted cell cycling in Alzheimer’s disease neurons and proposed that the ultimate death of the nerve cells is attributable to the genetic imbalance caused by the tetraploid status of their genome. In this study 400 hippocampal cells per patient were counted and subsequently examined manually.

Besides the possibility to measure the cell cycle with Slide Based Cytometry (SBC), this method offers the opportunity to perform full automated analysis of solid tissue. Therefore, the aim of this study was, to develop a method for the automated quantitative analysis of cell cycle in the neurons of AD patients. Most used method for the analysis of the cell cycle is Flow Cytometry but brain is solid tissue and converting the neurons into suspension for a measurement with Flow Cytometry (FCM) provides debris and loss of structural information.

It is a possibility to quantitatively analyze solid tissue sections by SBC instruments such as the LSC. The LSC provides the possibility to measure tissue sections and was therefore chosen to be the tool for the analysis of neurons from preserved brain tissue. The LSC was commercially introduced in the early 1990ies. It is a microscope-based instrument that might help to fill the gap between high throughput multiparametric cytometry on the one hand and morphological analysis and documentation on the other hand. Instrumentation and software of the LSC is explained elsewhere. The instrumentation is built around a routine epi-fluorescence microscope. It is equipped with a motorized stage that holds the microscopic slide. The specimen is immobilized on a conventional uncoated glass slide and analyzed by excitation with either one or two single laser beams. The setup of the LSC allows excitation with blue (488nm, argon laser) or red (630 nm, helium neon laser) light. The area on the glass slide which is measured is scanned stepwise. The emitted fluorescence light is guided to an optical bench equipped with four photomultipliers. With this equipment four fluorochromes can be distinguished with a single scan. Above all, we have recently presented data that show that with appropriate minor modification in the filter settings the instrument is able to detect and quantify up to six fluorochromes simultaneously. Moreover, the excitation light scattered by the specimen is detected by a photodiode below the slide producing a signal called forward scatter, a parameter related to cells morphology. After each scan step a digital image is created for each activated photomultiplier (PMT), i.e. for each fluorescence. Analysis can be performed with the appropriate software (Win Cyte™) according to the operators settings. A unique feature of the LSC is its function to review each cell of a measurement due to the x-y-position of each measured event. With this feature each cell can be visualized in light or fluorescence microscope manner and even its fluorescence can be analyzed during or after the measurement. With the aid of this function it is possible to verify measured data, particularly:

- If objects are single cells, doublets, debris or artifacts
- Document the cells morphology or fluorescence
For this study we used the special features of the LSC allowing to measure solid tissue on the slide and to verify the results. We herewith introduce a novel slide based approach for the standardized quantification of cell cycle in brain tissue that may be suited for diagnostical use in future.

2 Methodology

2.1 Patients and specimens

Brains of 13 patients with AD (mean age 70.4 ± 17.2 years) and nine controls dying without a history of neurological or psychiatric illness (66.1 ± 14.1 years) were obtained at autopsy. Each case of AD met the National Institute of Neurologic and Communicative Disorders and Stroke (NINCDS) and Alzheimer’s disease and Related Disorders Association (ADRDA) criteria for definite diagnosis of AD. All investigated AD cases were classified according to Braak and Braak (1991): Stages I-II: seven cases, Stages III-IV: two cases, Stages V-VI: five cases. The whole procedure of case recruitment and performing the autopsy was elaborated in accordance with the convention of the council of Europe on Human Rights and Biomedicine and had been approved by the responsible Ethical Committee of the University of Leipzig. Brains were fixed in 4% phosphate buffered formaldehyde (pH 7.4) and cryoprotected in 30% sucrose. Sections of 20/30µm thickness were cut from blocks of hippocampus and entorhinal cortex on a freezing microtome.

2.2 Immunohistochemistry

In order to find settings with the lowest coefficient of variation (CV) of DNA fluorescence, several sorts of slides (1. uncoated, Roth, Karlsruhe, Germany; 2. SuperFrost® Plus, Menzel, Bielefeld, Germany; 3. gelatine coated [1.4%vv gelatine and 0.05%vv KCr(SO$_4$)$_2$•12H$_2$O in aqua dest] slides) were tested with PI-stained leukocytes. Furthermore, frozen sections of several thicknesses (20 and 30µm) were analyzed to find the best settings for automatized measurements. 20µm was chosen for the LSC analysis.

Immunohistochemistry was performed as follows: Free-floating sections were pretreated with 50% formic acid in Tris-buffer (TB-T: 0.1M Tris, 0.15M NaCl, pH 7.6, 0.05% Tween 20 (SIGMA, Taufkirchen, Germany)) for 10 minutes. Nonspecific binding sites were blocked with TB-T containing 2% Normal Goat Serum (DAKO, Hamburg, Germany) and additional 1% Tween20. To identify neurons, the sections were incubated with a monoclonal antibody against the neuron-specific nuclear protein NeuN (1:500 diluted, Chemicon, Pittsburgh, PA, USA) at 4°C overnight. Immunoreaction was visualized using a biotinylated secondary goat-anti-mouse-antibody (1: 1000 diluted, Dianova, Hamburg, Germany) and the Cy$_2$-conjugated Streptavidin (1: 500 diluted, Dianova). For the amplification of the low cyclin B1 immunohistochemistry signal in the same sections the TSA™Biotin System (Perkin Elmer Life Sciences, Boston, MA, USA) was used. The polyclonal rabbit antibody against full length human cyclin B1 (clone H-433, diluted 1: 500, Santa Cruz) was applied in blocking buffer (0.1M Tris-HCL, 0.15M NaCl, pH 7.5, 0.5% blocking reagent) for an overnight incubation (4°C). Visualization was performed using biotinylated secondary goat-anti-rabbit antibody (diluted 1: 2,000) followed by an incubation with Streptavidin-HRP (diluted 1: 100), Biotinyl Tyramide Amplification reagent and Cy$_2$-conjugated Streptavidin (diluted 1: 500, Dianova). Subsequently floating sections were treated with 50µg/ml propidiumiodide (PI) in Tris buffered saline (0.01M Tris, 0.145M NaCl, pH 7.4) containing 100µg/ml Ribonuclease A (Sigma) for 30 minutes at 37°C. Sections were now mounted onto slides using DAKO® fluorescent mounting medium and stored at 4°C in the darkness until analysis.

2.3 Sample analysis

The microscope slide with the specimen was placed onto the motorized stage of the microscope. Measurements were performed with an LSC (CompuCyte, Corporation, Cambridge, USA). For excitation the 488nm line of the Argon (Forward scatter, Cy2 and PI) and the 633nm line of the Helium -Neon (HeNe) (Cy5) laser were used. Triggering signal for cell detection was the PI signal. Threshold mostly was set 2000. Analysis was performed with 20x objective, numeric appature 0.5. The minimum area to be necessary for a nucleus to be accepted was set to 8µm in order to exclude debris.
and other smaller artifacts (e.g. cut nuclei) from the analysis. 10 pixels were added to threshold in order to include cytoplasmic data. The following filters were used for the detection of fluorescence light from fluorochromes:

- Cy2: -530/dichroic filter (DF) 30
- Cy5 & PI: 650 EFLP

The data acquisition was started and the scan data images which were produced for the Forward scatter, Cy2, Cy5 and PI was checked for proper recognition of the software. If not all cells were recognized or too many cells were merged into one object the threshold value was changed accordingly (figure 1). Data acquisition was performed with the proprietary software of the LSC, WinCyte\textsuperscript{TM}, version 3.4. Using the software, a first window was created (Area vs. DNA-PI Integral). In this window a gate was set to exclude artifacts, debris and doublets (figure 2a). Singletons were gated into a second window (DNA-PI Max Pixel vs. DNA-PI Integral, figure 2b). From this window cells were gated into a third window in order to exclude further doublets (Perimeter\textsuperscript{2} vs. DNA-PI Integral, figure 2c). With the perimeter\textsuperscript{2} feature it was possible to exclude further doublets and multiple events. Cells were gated into a fourth window (Cyclin B Max Pixel vs. NeuN-Cy5 Max Pixel, figure 2d). After creating regions with cells of neuronal and non-neuronal origin, each region was gated separately into a window with DNA-PI Integral vs. count (data not shown).

As a control, a specimen of each patient was measured with only the secondary antibody by omitting the primary antibody.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Triggering on nuclear DNA with different threshold levels.}
\end{figure}

Images show the scan data images acquired during analysis of brain sections stained with PI

a) DNA-PI data (no triggering)
b) DNA-PI; trigger=PI; threshold 800
c) DNA-PI; trigger=PI; threshold 1200
d) DNA-PI; trigger=PI; threshold 1600
After finishing the analysis, the PI data was exported into an *.fcs-file for the cell cycle analysis by ModfitLT for Mac version 2.0 (Verity software house, Inc. Topsham, ME, USA).

### 3 Results

Following evaluation of staining and microscope slide coating was performed before automizing the brain section analysis by LSC:

A. PI-stained leukocytes were measured on several slides to detect the method with the lowest CV of DNA-PI fluorescence: uncoated slides were found to be best suited for the analysis since CV was the lowest in contrast to
other used slides (figure 3). The reason of the broader CV value in the case of gelatine coated slides is the higher background staining due to the stronger binding of PI through its positive charge and that DNA is a possible constituent of gelatine.

B. Frozen brain sections were analyzed in several thicknesses: 20-30µm thickness. 20µm was chosen for the LSC analysis.

The following settings of the LSC were found to be the best to unequivocally differentiate between cells of neuronal and non-neuronal origin as well as for the best detection of Cy2 and the quantification of PI: trigger: PI, threshold: mostly 1200 (figure 1), this was verified and adjusted if necessary prior to analysis in order to provide exact results. Minimum area: 8µm². Add pixels to threshold: 10. Scatter was measured in the Scatter channel of the Ar laser (offset: 2,092, gain: 18), CyclinB-Cy2 was measured in the green channel of the Ar laser (PMT: 10%, offset: 2,075, gain: 255), PI in the red channel of the Ar laser (PMT: 30%, offset: 2,075, gain: 255) and Cy5 in the red channel of the HeNe laser (PMT: 45%, offset: 2,073, gain: 255).

PI, Cy2 and Cy5 fluorescences could be detected with the LSC, thus it was possible to differentiate between neurons (NeuN-Cy5), dysregulation in the cell cycle (CyclinB-Cy2) and nuclear staining (PI). Since PI was chosen to be the trigger, false positive events (e.g. cells without nucleus) were excluded on the one hand. On the other hand, the cell cycle could be depicted with the PI fluorescence (figure 4). Due to the feature of the LSC to document the morphology it was possible to differentiate between pyramidal cells and other subpopulations of neuronal origin (figure 5).

Scatter was insufficient to analyze the morphology since the Forward scatter signal of the tissue did not allow to differentiate single cells (figure 6).

An automated quantification of cell cycle and aneuploidy is possible with LSC. The amount of DNA as well as the cell cycle can be examined.

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**Figure 3: Detection of a slide with lowest CV by measuring PI (50µg/ml) stained leukocytes**

- **a:** x-y-position of the analyzed events on the slide
- **b:** Gating by excluding debris (left from the polygon) and doublets (right from the polygon)
- **c:** uncoated slide
- **d:** SuperFrost®Plus slide
- **e:** gelatine coated slide

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cells in this window are gated out of the upper half of figure 2d (cells declared as neurons due to NeuN-Cy5 fluorescence). Cells in area 1 were declared as G0/G1. Cells in area 2 and 3 proved to have higher DNA content. In area 3 cells have the twofold DNA content as cells in area 1.
4 Discussion

Recent studies showed that neurons of AD patients initiate their death by re-entering the cell cycle\textsuperscript{1,3,6}. With the LSC it was possible to show cell cycle in cells of brain tissue which were unequivocally identified as neurons. If there are differences between AD patients and control brains remains to be verified with further examinations. However, the analysis of brain tissue by SBC for determining the cell cycle requires knowledge of the analyzer concerning the LSC and its software. For example it is important to “add pixels to threshold” to avoid that only data of the nuclei are gained. By ignoring the cytoplasm, no or little fluorescence information of the cytoplasm (Cy2 and Cy5) is gained. Comparing to other tissues the density of nuclei is low in the brain tissue but because 20µm thick sections

\textbf{Figure 5: CompuColor picture of the stained human entorhinal cortex}

NeuN-Cy5 was defined to be green, DNA-PI was defined to be red in this overlay image of a scanned brain tissue section.

One can see that there are many cells NeuN negative, probably glial cells.
nuclear overlapping could be observed. Thus, many doublets will be triggered. The user of the LSC has to minimize the count of triggered doublets by changing the threshold on the one hand. The higher the threshold is chosen, the less doublets will be measured. The lower the threshold is chosen, the less cells are ignored but the more doublets and multiple events will be triggered. On the other hand it has to be taken care, that all events with nucleus are triggered. Events which are not triggered can not be included into the statistics nor is it possible to relocate them for a further morphological evaluation and documentation.

After finishing the remeasurement and acquisition one has to exclude the doublets from the statistics. This can be performed by relocating events of a certain cell area which is thought to be too big for a single nucleus. If the relocated event is actually a doublet one can exclude each event of this area by gating for the singlets.

To the best of our knowledge this is the first study providing an automized method for the analysis of cell cycle in solid brain tissue. The thickness of the slices must be chosen very carefully to diminish contortion of the PI signal (DNA content). If slices were too thin (<20µm), most of the nuclei will be cut and if slices were too thick (>30µm), the user could not distinguish cells lying on top of each other. Therefore a thickness of 20µm proved to be most suitable for the brain sections.

Figure 6: Morphology and fluorescence of the brain sections as seen by the LSC.

a) CyclinB-Cy2 fluorescence (dysregulation of the cell cycle). Excited with Ar laser, measured in PMT1
b) PI-DNA fluorescence (nuclear staining). Excited with Ar laser, measured in PMT3

Figure 6: Morphology and fluorescence of the brain sections as seen by the LSC.

a) CyclinB-Cy2 fluorescence (dysregulation of the cell cycle). Excited with Ar laser, measured in PMT1
b) PI-DNA fluorescence (nuclear staining). Excited with Ar laser, measured in PMT3
c) NeuN-Cy5 fluorescence (neuronal marker). Excited with the HeNe laser, measured in PMT3
d) Scatter fluorescence: note that it is not possible to distinguish between several single cell events
Within the automated SBC analysis of the brain tissue it was possible to unequivocally differentiate between PI, Cy2 and Cy5. Thus it is possible to depict the cell cycle (PI integral) of NeuN\(^+\) and NeuN\(^-\) cells. Moreover Cyclin B content of NeuN\(^+\) and NeuN\(^-\) cells could be judged. The possibility of the LSC to perform morphological documentation allows e.g. a differentiation between pyramidal cells and other neuronal subpopulations. This allows (at least combined with the immunophenotyping) a limited information about morphological structures (figure 5). In contrast a morphological documentation only by scatter fluorescence was not possible. A bright forward scatter signal of the tissue did not allow to detect single cells in this channel.

In our present experience the LSC is very useful for the analysis of cell cycle in brain tissue for several reasons. On the one hand the examination of PI and thus the depiction of the cell cycle is possible. Furthermore, more neurons were counted with this method than with any other described method so that statistical evaluations are more reliable. Of course, LSC is a more sophisticated way to quantitate cell cycle activity in neurons than with manual scoring of arbitrary fields on the slide. We regularly found, that distribution of cells on the slide is inhomogenous and therefore the whole region of interest must be analyzed.

Therefore we suggest LSC as a routine tool for the quantification of cell cycle activity in neurons of patients with AD. In future experiments we propose to use the LSC in tissue sections of patients with AD in order to see differences in cell cycle activity between patients with differing severeness and endurance.

By these studies we hope to demonstrate the power of the LSC for the routine pathological use. This should add up to the bright versatility of applications for the LSC as a slide based cytometric instrument\(^{19,22-24}\).

**Acknowledgement**

We like to thank the interdisciplinary center for clinical research (IZKF) for financial support of the study


**Literature**


