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# Assessment of X Chromosome Centromere Instability in Alzheimer's Disease: A Quantitative FISH Approach

**Neuron X Chromosome Centromere Instability in AD**

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**Abstract**: Chromosome instability in Alzheimer's disease (AD) neurons has been previously reported. This pilot study aimed to establish a quantitative technique for assessing X chromosome centromere signals using fluorescence in situ hybridization (FISH). Hippocampal brain tissue was collected at autopsy from sporadic AD patients and age-and gender-matched controls. FISH was utilized to detect and measure the intensity of hybridization signals for X chromosome centromeres in the interphase nuclei of hippocampal brain cells. The premature centromere division (PCD) phenomenon, marked by a close bipartite signal appearing as two separated FISH spots, was examined to see if the hybridized DNA amount in each spot matched the expected centromere DNA amount. The technique effectively distinguished between PCD+ and PCD- signals. The average frequency of PCD,X in the AD group was 7 ± 1%, compared to 3.2 ± 0.84% in controls. This quantitative approach supports qualitative analyses of FISH centromere spots, reinforcing findings of chromosomal instability in AD. The presence of a double signal at the centromere of a single X chromosome indicates re-entered cell cycles, DNA replication, and PCD in hippocampal neurons. This technique provides a reliable method for identifying PCD + signals and contributes to understanding chromosomal instability in AD.

**Keywords:** Alzheimer's disease; premature centromere division; X chromosome; chromosome instability; fluorescence in situ hybridization

## 1. Introduction

Alzheimer's disease (AD) is a complex and progressive age-related neurodegenerative disorder. The cytopathology of AD is very complex. Most age-related neurodegenerative diseases present different brain pathologies and clinical features, but they all share a common trait: a reduced number of neurons in specific parts of the brain [1]. The identification of AD biomarkers is a highly relevant research area, and many different approaches are being explored [2]. To date, no reliable biomarker has been established that enables the diagnosis and/or assessment of the degree of risk of AD in susceptible individuals. Understanding how the loss of neurons occurs and which cytopathological processes precede it remains a major challenge for researchers.

One potential cell mechanism is the generation of neuronal aneuploidy as a consequence of neuron cell cycle reentry. Evidence suggests that chromosome malsegregation is related to

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neurodegeneration in AD patients [1,3–6]. A significant increase in chromosome instability (CIN), as well as the presence of aneuploidy and premature centromere division (PCD), has been observed in AD brain neurons and peripheral tissues [6–9]. Chromosomal instability, especially alterations of the cell cycle and chromosome segregation patterns, may be a key feature leading to the apoptotic degradation of neurons in AD [9–12].

Several studies have reported that X chromosome alterations indicate malsegregation during cell cycle events in AD patients [7–9]. Loss of control over centromere separation is a potential cause of improper chromosomal segregation, closely related to aneuploidy [9]. It has been shown that the PCD of the X chromosome is substantially present in the interphase nuclei of frontal cerebral cortex neurons of AD brains, as detected by the FISH method, and manifested as close paired fluorescent dots [8].

Given the frequent occurrence of X chromosome alterations in AD, it is beneficial to implement new approaches for assessing X chromosome imbalances (and other chromosomes) in interphase nuclei. Fluorescence in situ hybridization (FISH) targeting the centromere region of the X chromosome is the method of choice for verifying centromere instability in interphase nuclei.

FISH targeting the centromere region of the X chromosome was applied to the interphase nuclei of hippocampus tissue from sporadic AD women, post mortem. For the quantitative analyses of FISH signals, an image analysis system was used to determine whether duplicated centromere spot signals represent the PCD phenomenon. To the best of our knowledge, this is the first time quantitative analysis has been performed on post mortem brain samples to determine centromere instability in the neurons of AD patients.

## 2. Material and Methods

### *2.1. Subjects*

Hippocampal brain tissue was collected at autopsy from five sporadic female AD patients (ages 74–79 years) and five age-matched female controls (ages 73–78 years) according to approved protocols and with written family consent. Permission from the Ethics Committee of the Faculty of Medicine, Belgrade University, was obtained.

### *2.2. FISH Analysis of Premature Centromere Division on the X Chromosome in Interphase Nuclei*

Brain tissue was routinely formalin-fixed, paraffin-embedded, and sectioned at 4 μm. Histological preparation of hippocampus sections embedded in paraffin was performed by immersing the sections into xylene (Sigma-Aldrich, Munich, Germany) at room temperature. Sections were rehydrated in a series of ethanols (100% to 70%), then washed in phosphate saline buffer (PBS) pH 7.4 (Sigma-Aldrich, Munich, Germany). The slides were immersed into $H_4BNa$ 10 mg/ml PBS, followed by washing in PBS, deionized water ($deH_2O$), and 0.1 N hydrochloric acid. After neutralization with $deH_2O$, preparations were immersed into 2xSSC buffer (Sigma-Aldrich, Munich, Germany) and 8% (v/v) NaSCN at 80°C. Slides were then rinsed with deH2O and 2xSSC buffer before being immersed into a trypsin solution (1 mg/ml saline solution) at 37°C. Trypsin activity was blocked by immersion into 0.5% newborn calf serum solution (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Slides were dehydrated in a series of ethanols (70% to 100%) and air-dried. Cytocell (UK) directly-labeled (FITC) $\alpha$-satellite pancentromeric probes for the X chromosome were applied to preparations. Manufacturer's co-denaturation and hybridization instructions were followed. After hybridization, slides were washed with 2xSSC 0.05% Tween (Sigma-Aldrich, Munich, Germany), dehydrated in a series of ethanol, and counterstained with DAPI-Antifade (Life Technologies, Eugene, Oregon, USA).

Slides were analyzed using an Olympus AX50 epifluorescent microscope (Olympus, Tokyo, Japan) under 1,000x magnification. The intensity of centromere signals in neurons was determined using Cytovision 3.0 software (Applied Imaging, Hampshire, UK). The intensity of single-spot signals and split-spot signals indicating PCD was measured separately. Based on the value of measured intensities, it was concluded whether a duplicated signal represented PCD. If the intensity

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of fluorescence of a double-spot signal corresponded to twofold the intensity of a single centromeric signal, the two neighboring dots were considered as a visual manifestation of PCD.

*2.3. Statistical Analysis*

Statistical analysis was performed using the Mann–Whitney test with Statgraph 4.2 software.

**3. Results**

The relative fluorescence intensity of centromere signals in neuron cells was measured. In females, the diploid neuronal nuclei exhibited two distinct FISH spots for the centromere of each X chromosome, showing that the signals were of similar fluorescence intensity. Nevertheless, in some cells, split signals for one centromere were observed. The fluorescence intensity of each divided FISH spot was similar to the undivided centromere signal. If the fluorescence intensity of the split signals was twice that of the single centromere signal, we concluded that the PCD phenomenon was present in the observed cells (PCD+) (Figures 1–4). Conversely, if the joint fluorescence intensity of the divided signals was equal to the fluorescence of a single centromere signal, we represented this as the absence of PCD (PCD-)(Figure 5).

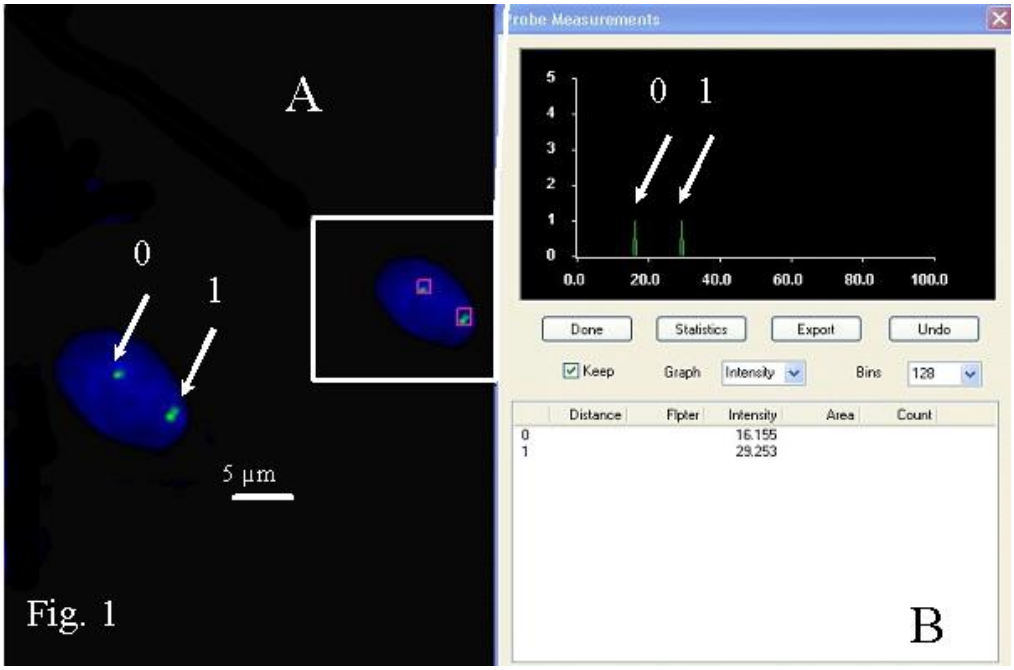


Fig. 1

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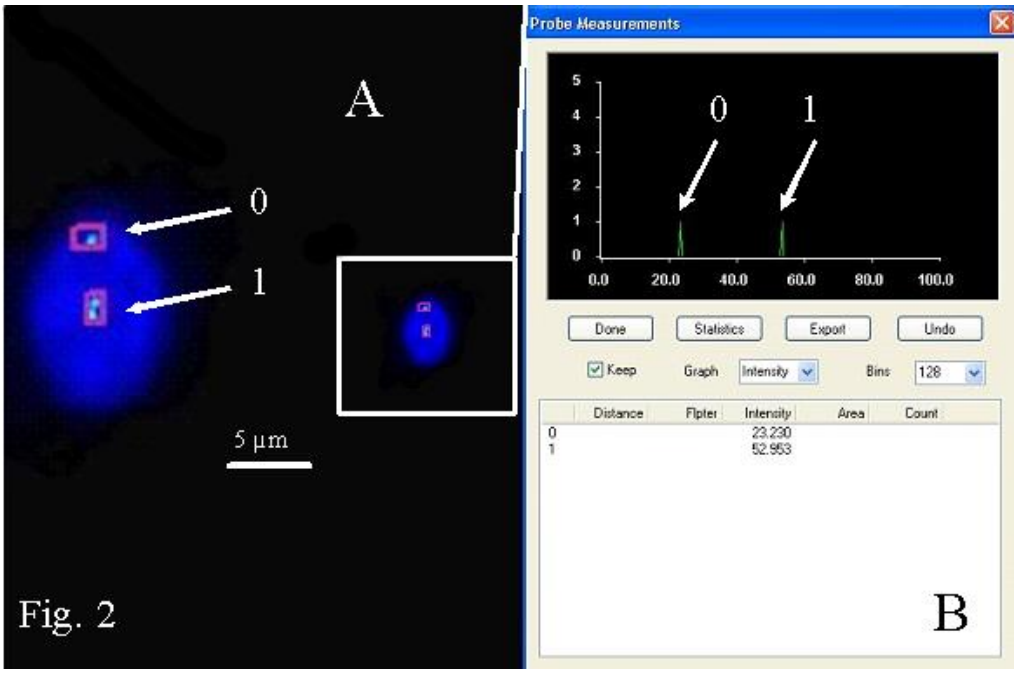


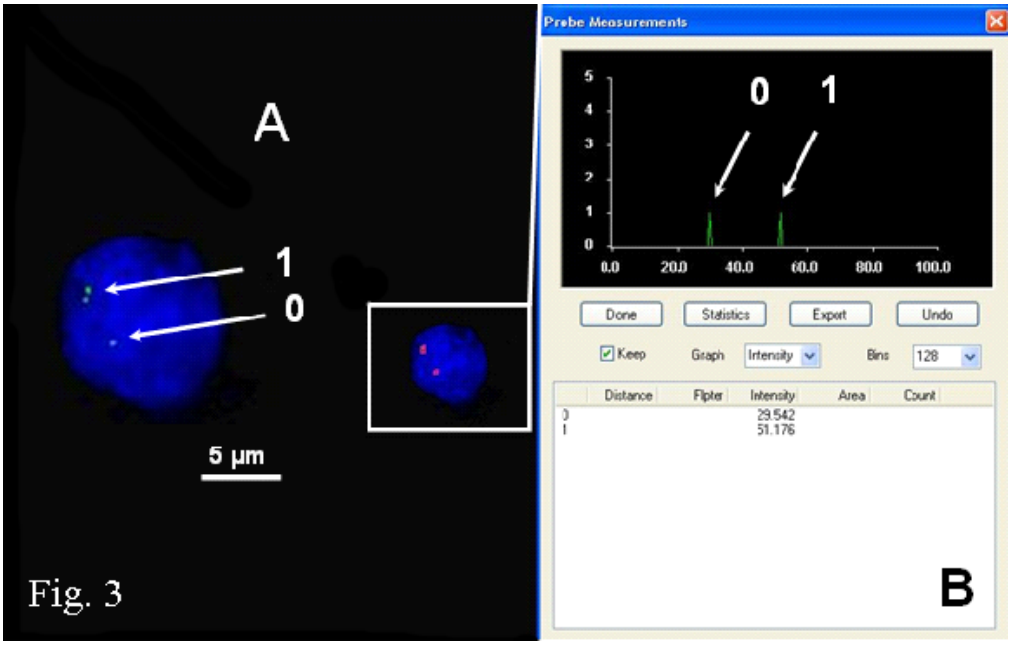


**Figure 1–3.** Examples of quantitative assessment of FISH signals. (A) Nucleus with one spot signal of a specific probe for chromosome X (relative intensity – arrow 0) (PCD-) and two spots (PCD+), chromosome X-specific probe (arrow 1). (B) Relative intensity of each signal presented by the DNA histogram obtained by measuring nuclear DNA content.

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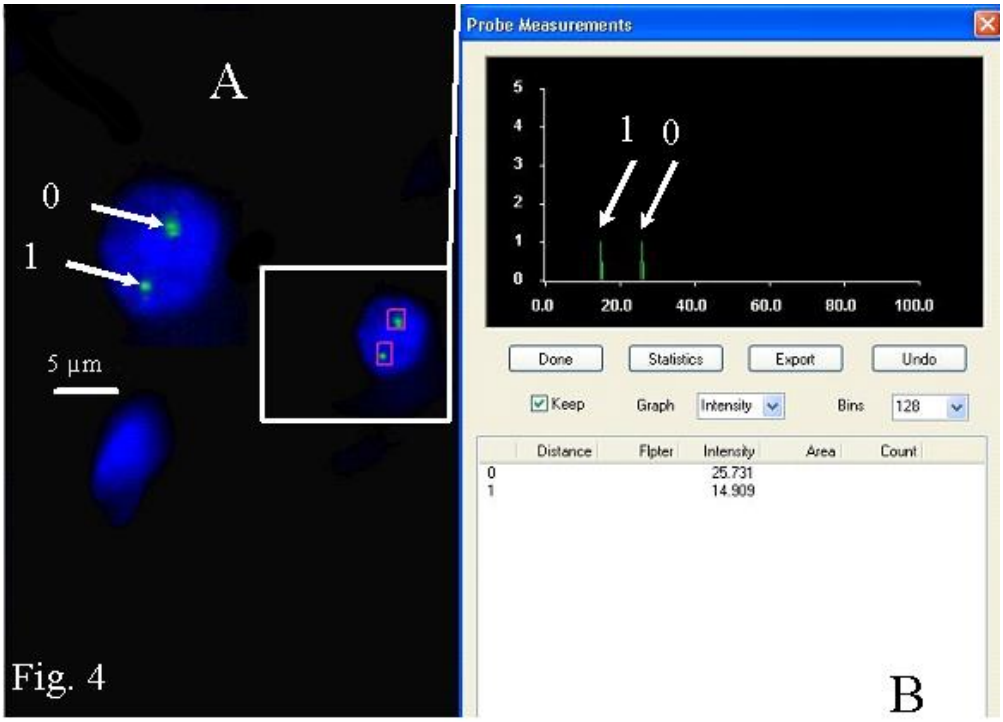


Fig. 4

**Figure 4.** Example of a split signal detected by quantitative assessment of FISH signals. (A) Nucleus with one spot signal of chromosome X-specific probe (relative intensity – arrow 1) (PCD-) and two spots (PCD+), chromosome X-specific probe (arrow 0). (B) Relative intensity of each signal presented on DNA histogram obtained by measuring nuclear DNA content.

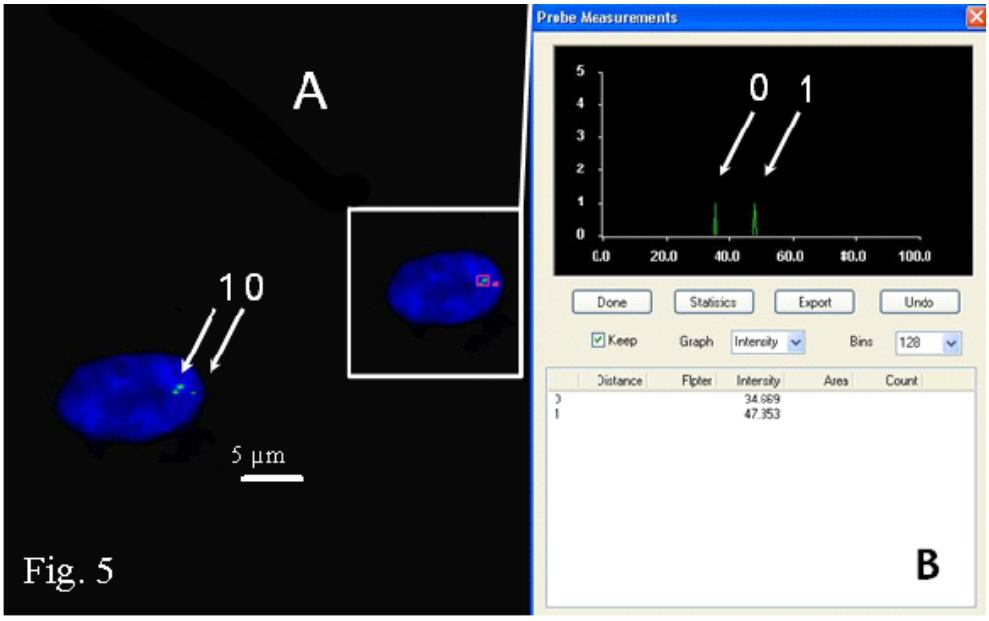


Fig. 5

**Figure 5.** Example of a split signal detected by quantitative assessment of FISH signals. (A) In AD brain neuron cell counterstained with DAPI with signals of chromosome X (PCD-) and two spots like chromosome X-specific probe (relative intensity of each spot in pixels) characterizing split signals in the centromere. (B) DNA histogram obtained by measuring nuclear DNA content, revealing that both signals 0 and 1 have similar fluorescence intensity, indicating that signal 1 represents a split signal rather than PCD.

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After analyzing neuronal nuclei from all AD and control samples, the average frequency of PCD,X in the AD group was found to be 7 ± 1%, whereas, in the group of five age-matched female controls, the average frequency of PCD,X was found to be 3.2 ± 0.84% (Figure 6). In both AD and control cases, PCD,X was present on only one of the two X chromosomes in all analyzed nuclei. The results show an almost twofold higher incidence ($p<0.1$) of PCD,X in hippocampal neurons of AD patients compared to age-matched controls.

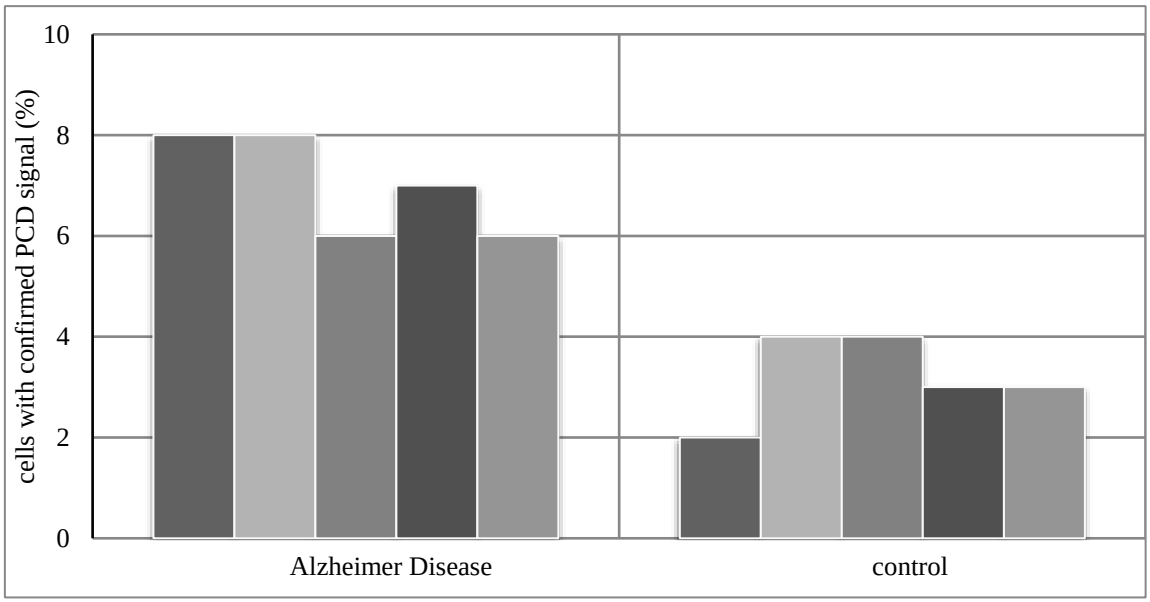


**Figure 6.** Quantitative assessment of X chromosome FISH signals in interphase nuclei. AD and control cells with confirmed PCD signals. One hundred interphase nuclei from hippocampal neurons were scored for each sample. The percentage of nuclei displaying PCD,X is significantly higher in the AD cases analyzed compared to controls ($p < 0.01$; Mann–Whitney).

## 4. Discussion

The assessment of FISH spots is a cumbersome process [13–18], especially when estimating the PCD phenomenon. Therefore, a novel approach to analyzing FISH spots in chromosome imbalances would be welcomed. Until now, the fluorescence intensity of hybridization (centromeric) spots in chromosomal alterations has not been substantially investigated. Using DNA FISH probes that hybridize to the alpha satellite DNA of the centromeric region provides an advantage for detecting centromeric chromosome (DNA) copy numbers in interphase nuclei [10,13–15,18], evaluating human interphase chromosome instability (CIN) in brain nuclei, as well as peripheral tissue cells.

Fukagawa et al. [19] defined the PCD phenomenon in interphase nuclei as a bipartite centromere signal if the distance between two signals was not greater than half of the centromere width, while a single dot-like signal was scored as PCD-. To properly address X chromosome variation of CIN in interphase nuclei, the key question is: how can we easily and accurately estimate the PCD phenomenon using FISH for the centromeric region, and establish a clear distinction regarding chromosomal aneuploidy?

In this study, we present an image system that allows quantitative analysis of the fluorescence intensity of the centromere region, i.e., the size of individual FISH spots. Since the PCD phenomenon is characterized as a bipartite signal with two separated FISH spots, it is useful to assess whether the quantity of hybridized alpha satellite probe in each spot corresponds to the expected centromere amount. This approach allows us to make a clear distinction between the PCD phenomenon and split centromere signals. Split centromere signals contain mainly singles with low copy number (low intensity), indicating that the bipartite signal stands for a split signal.

Variations of alpha satellite DNA presented in the centromeric regions of all human chromosomes are detectable by quantitative analysis of FISH for chromosome-specific alphoid DNA probes [20,21]. This approach revealed that one of two centromeric spots in a CAL-51 breast cancer

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cell was apparently brighter than the other when homologous chromosomes 7, 11, 17, and 18 were analyzed [22]. This variation in FISH signal fluorescence intensity of spots on the two homologous chromosomes in the nucleus may result from differences in the origin of homologous chromosomes (paternal and maternal). Furthermore, the quantitative approach indicates that genetic changes in the cell cycle can be assessed by estimating the fluorescent intensity of FISH spots. The intensity of centromeric FISH spots increases during the S phase, displaying a significant increase in the late S phase, similar to that from the G2 phase, showing that alpha satellite DNA is replicated in the late S phase [22].

Moreover, interphase nuclei of neurons had one signal per nucleus after hybridization with classical satellite DNA probes for heterochromatic regions of chromosomes 1, 9, and 16 in 5% to 40% of brain nuclei [23]. Quantitative assessment of FISH signals in interphase cells with one signal displayed approximately two-fold intensity and represented the nuclei with two somatically paired (or over-posed) FISH signals on both homologous chromosomes. These assessments indicated that the mentioned nuclei could not be considered to be monosomic.

Additionally, Iourov et al. [23] presented the quantitative assessment of variable X chromosome FISH signals, allowing for the differentiation of homologous X chromosomes into active and inactive by alphoid DNA heteromorphism. This approach could be helpful in X chromosome inactivation studies in females with mosaic forms of X chromosome aneuploidy.

In this study, the micro image system was used to estimate the intensity of FISH spots for the X chromosome centromere region (alpha satellite DNA of the centromeric region) by conducting quantitative analysis. This approach can be considered an efficient tool for the accurate and easy determination of CIN in AD interphase nuclei. To the best of our knowledge, this is the first time the analysis of the FISH spot intensity of the centromere region was used to determine the PCD phenomenon in AD brain cells. Based on our comprehensive observations, we conclude that a quantitative approach should support the qualitative analysis of FISH centromeric spots in the study of chromosomal alterations in AD. The mere presence of a double signal at the centromere of a single X chromosome is proof that hippocampal neurons have re-entered the cell cycle, DNA has replicated, and premature centromere division has occurred.

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**Author Contributions:** Conceptualization, B.S.P. and V.B.; methodology and formal analysis, V.B., L.Ž. and B.S.P.; investigation, L.Ž. and B.S.P.; data curation and visualization, D.T. and P.P.; writing—original draft preparation, L.Ž., D.Ž., P.P. and D.T., writing—review and editing, B.S.P., V.B. and G.P. All authors have read and agreed to the published version of the manuscript.

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