SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D.)

Development of cytometric methods for the detection of genetic and epigenetic changes

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The Examination takes place at Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen
Debrecen; at 11 AM, 28th of June, 2019.

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Debrecen; at 1 PM, 28th of June, 2019.

PREMISES

Development of new methods may lead to novel observations or a deeper understanding of phenomena already observed. Novel approaches include hitherto untested combinations of known techniques and their application for new purposes. Our lab has got a longstanding interest in developing novel methodical platforms based on the creative combination of cytometric methods with molecular biological technology. This strategy has been successfully applied in the development of various lab-on-beads tools and also to answer basic biological questions related to global aspects of nuclear structure and chromatin architecture. My Ph.D. program fits this research strategy: I have explored and further exploited the potentials of cytometry in genetic and epigenetic research, for possible diagnostic applications and in support of ongoing basic research projects related to chromatin structure. I have developed new flow cytometric microbead assays what can be used for the diagnosis of certain genetic diseases, and flow and laser scanning cytometric techniques to address global epigenetic features.

SPECIFIC AIMS

Exploring the possibilities offered by flow cytometry for the detection of genetic alterations, our aim was:

A - to develop methods to detect insertions, deletions, triplet expansions and point mutations by microbead based flow cytometric analyzes.

Exploring the possibilities offered by laser scanning cytometry and flow cytometry for global epigenetic analyzes, our aim was:

B - to develop *in situ* methods to assess intrinsic or superhelicity dependent nucleosome stability, a chromatin feature of regulatory significance, in a post-translational modification and histone subtype dependent manner and with special emphasis of possible long-range effects of changes in the superhelical state of the chromatin.

C - to develop microbead-based assays to detect the presence of different epigenetic traits in a particular chromatin context.

A. CYTOMETRIC MICROBEAD ASSAYS FOR GENETIC ANALYZIS

A. 1. INTRODUCTION

Flow cytometric platforms offer possibilities for the investigation of populations of many types of particulate material (e.g.: live or fixed cells, beads), measuring different parameters (e.g.: side scatter, forward scatter, fluorescence intensity) in a multiplex and high-throughput manner. In conjunction with microbead analyzes, flow cytometry has proved to be a sensitive and versatile platform of biochemical, immunological, or molecular biological investigation of proteins and nucleic acids.

I tested and demonstrated the utility of two novel microbead based strategies. On the one hand, I asked if *dependence of the melting temperature* of double-stranded (ds) DNA molecules could be exploited to detect various genetic alterations. Above its T_m, a sample containing a PCR product prepared using a pair of biotinylated and fluorescent primers and immobilized on microbeads via the biotin moiety, will dissociate into free single-stranded (ss) DNA molecules labeled with the fluorescent tag and the bead-attached, non-fluorescent, biotinylated complementary strand. The degree of denaturation, i.e. ratio of the ds and ss species at equilibrium, will depend on the incubation temperature relative to the T_m. To aid the implementation of the method, melting temperature can be reduced to near ambient conditions in the presence of H-bond destabilizing reagents such as DMSO or formamide. In view of the fact that the degree of denaturation at a given temperature is expected to depend also on the length of the DNA molecule, measurement of the above ratio by flow cytometry was used for the length comparison of PCR products prepared from DNA samples in diseases where this approach might be of diagnostic value.

In another experimental strategy, I tested if I can detect single-stranded regions in heteroduplices formed after denaturation and reannealing of different size PCR products, using restriction enzymes unable to cleave their recognition motives in the single-stranded overhang area. When a deletion or rearrangement due to chromosome translocation removes a particular restriction enzyme recognition site, the PCR products of the normal and pathological sample yield homo- and heteroduplices what can be distinguished based on the presence or absence of the recognition site. I used streptavidinated microbeads anchoring the fluorescently tagged hybrids through their biotinylated ends.

A. 2. RESULTS

A. 2. 1. Detection of CAG triplet expansion in Huntington's disease

The CAG repeats encode a polyglutamine region at the N-terminal domain of the huntigtin protein that is a key player in Huntington's disease. It is known that the developement of the disease correlates with the lenght of the CAG triplets. There are four categories based on the number of CAG-repetitions: healthy (<27 CAG), and intermediate (27-35 CAG) are not associated with the manifestation of symptoms, but occasionally fathers with repeats in the latter range will transmit the repeat to their children what is then expanded to the pathological range. From among those with reduced penetrance (35-39 CAG), some individuals will develop Huntigton's disease, while others will not. In the diseased phenotype (>40 CAG), symptoms appear at the age between 35 and 50 and correlation can be observed between the CAG repeat length and the age of onset. People with repeats of 60 or larger commonly have very young onset, i.e. before the age of 20.

The flow cytometric melting point analyzis was tested using a model system including a set of plasmids containing exon 1 of the Huntingtin (IT15/HTT/HD) gene harbouring different length CAG repeats. The assay could clearly distinguish between the templates representing normal and pathological triplet expansions in the case of this model experiment representing homozygotes and heterozygotes as well, irrespective of the fluorescent labels used. Next, genomic DNA isolated from lymphocytes of healthy volunteers, and anonymous patients suffering from Huntington's disease with predetermined number of CAG repeats were amplified and analyzed. All the samples with CAG repeat numbers in the healthy range gave low average fluorescence values as compared to the samples derived from people afflicted with the disease.

A. 2. 2. Detection of BRCA1 5382insC mutation

The BRCA1 point mutations predispose women to breast and ovarian cancer. From among these BRCA1 mutations the 185delAG, 5382insC and the missense C61G are the most common.

For the detection of BRCA1 5382insC, a modified allele specific PCR reaction was used, applying three primers in a multiplex reaction: wild type and mutation specific forward primers and a common reverse primer. Due to the presence of an extension sequence (CAG tag) at the 5' end of the mutation specific forward primer, two different length PCR products are generated if the analyzed sample is heterozygous for the mutation; thus the mutant and the wild type alleles can be discriminated using our assay. Following the optimization of the

formamide concentration, a clinical sample with determined heterozygous BRCA1 5382insC mutation and the control DNA of Jurkat cells could be clearly distinguished by flow cytometer.

A. 2.3. Detection of single-stranded DNA regions

When two, partially complementary strands anneal, the non-complementary regions stay single-stranded. PCR products representing such a model were exposed to a restriction endonuclease that can cleave the ds DNA carrying the target sequence but not ss DNA and the samples were bound to streptavidinated microbeads followed by flow cytometric analyzis. We observed a significant decrease of fluorescence only in the case of the microbead bound homoduplexes, just as expected for these ds specific enzymes.

A. 3. DISCUSSION

The microbead based methods described can be applied for the diagnosis of several genetic disordes where the length or the sequence of a certain genomic segment is changed. Genetic disorders that can be detected include triplet expansions, SNP-s, point mutations, microsatellite polymorphisms, insertion/deletion polymorphisms and rearrangements.

Microbead-based melting point analyzes was applied successfully both for the detection of the rare Huntington’s disease and the frequent 5382insC mutation of BRCA1 gene.

B. QUANTITATIVE IMAGING CYTOMETRY FOR EPIGENETIC ANALYZIS

B. 1. INTRODUCTION

Histones are the fundamental units of nucleosome structure in which a histone octamer consisting of four different types of core histones is wrapped around by 146 bp long stretches of the genomic DNA forming the first level of chromatin compactation. The octamer is composed of two H2A-H2B dimers and one $(H3\text{-}H4)_2$ histone tetramer. Neighbouring nucleosome core particles are connected by the linker DNA which is associated with the linker histone (H1). Nucleosomes play essential structural and functional roles in the transition between transcriptionally active and inactive chromatin states. Stability of nucleosomes is of regulatory importance in eukaryotes since the formation of nucleosome free regions (NFRs) is a prerequisite for downstream steps of transcriptional activation. Histone–DNA and nucleosome-nucleosome interactions are affected by histone post-translational modifications (PTMs) that mainly occur on the N- or C-terminal tail of core histone molecules.

Although histones have a high degree of conservation, variants have evolved to assume diverse roles in gene regulation. Beside PTMs, histone variants can also have direct effect on nucleosome stability.

We have developed an assay based on quantitative imaging cytometry which delivers histone type and post-translational modification (PTM-) specific information on the stability features of nucleosomes consisting of native endogenous or ectopically expressed histones as well, *in situ*, in the individual nuclei of a cell population, and what is amenable to high throughput studies. In our method, agarose embedded cells are lysed and exposed to salt or to DNA intercalating agents, and the remaining chromatin-bound histones are detected using specific antibodies and quantitative microscopy conveniently performed by LSC (laser scanning cytometer), hence the name coined for the method: Quantitative Imaging of Nuclei after Elution with Salt/Intercalators (QINESIn).

B. 2. RESULTS

B. 2. 1. PTM-dependence of nucleosome stability

H3K4me3 carrying promoter-proximal nucleosomes were more sensitive to doxorubicin treatment than bulk H3 histones. Eviction of H3K4me3 was confirmed by elution with doxorubicin analyzed in parallel by LSC and ChIP-Seq (chromatin immunoprecipitation sequencing) in mES (mouse embrionic stem cells) cells.

GFP-tagged versions of histones H2B and H3 also exhibited differential doxorubicin sensitivity.

The differential eviction of H3K4me3 and H3K27me3-containing nucleosomes reported earlier could also be reproduced by QINESIn in intercalator elution using ethidium-bromide (EBr).

Next, we tested if the well-known differential dissociation of the H2A-H2B dimer vs. the $(H3\text{-}H4)_2$ tetramer from the nucleosome by *salt* could also be monitored by the QINESIn platform. The assay was able to clearly distinguish between these two histone complexes.

B. 2. 2. Effect of histone variants on nucleosome stability

To investigate the effect of histone variants on nucleosome stability, first H2A.Z containing nucleosomes were analyzed by QINESIn using anti-H2A.Z antibodies from different manufacturers. (Those on H2A.Z below are unpublished data.) The two antibodies that were used to measure H2A.Z eviction gave different results in the salt elution assay. Surprisingly, the elution profile of H2A.Z, detected by an anti-H2A.Z antibody from Abcam was similar to that obtained for bulk H3, while the other antibody purchased from Thermo Fisher Scientific (TFS) revealed H2A-like stability features.

To test if the two antibodies might recognize different isoforms of H2A.Z, plasmid-derived, transfected H2A.Z isoforms expressed as fluorescent fusion proteins and endogenous, native H2A.Z1 and H2A.Z2 histones using H2A.Z1 KO or H2A.Z2 KO cell lines were analyzed. The Abcam antibody recognized both Z1 and Z2 isoforms and no difference could be detected in the elution profiles.

In further experiments, I tested H2A.Z mutants in which (1) the C-terminal tail was deleted (ΔC) removing the binding site of the H2A.Z reader protein PWWP2A, or (2) the lysines of the N-terminal tail that can be acetylated were changed to arginine (5KR). These mutant histones were expressed in H2A.Z1/Z2 double knock out cells. The salt elution curve of the ΔC mutant was shifted to the left, showing that the H2A.Z ΔC containing dimers become as destabilized as the canonical dimers (expressed in a replication dependent manner). Based on these data, PWWP2A has a major role in the stabilization of nucleosomes containing H2A.Z. Measuring the salt elution curve of the 5KR mutant lacking acetylation had no effect on nucleosome stability.

Using EBr as a destabilizing agent, the H2A.Z (detected by Abcam) eluton profile was similar to that of the canonical H2B or H2A. However, a major intercalator-resistant fraction

remained. The level of H2A.Z remaining after intercalator treatment was also PWWP2A-dependent.

The intercalator resistant fraction was further characterized after elution by high salt. SDS-PAGE was performed and the resultant bands were analyzed by MS.

The histone variants H2A.X and γH2A.X (phosphorylated H2A.X) were also analyzed by QINESIn. Significant destabilization of nucleosomes containing H2A.X was observed in salt elution but not in EBr elution experiments following phosphorylation of the histone variant by brief exposure of cells to etoposide.

B. 2. 3. Superhelicity dependence of nucleosome stability

Our method proved to be highly instructive in demonstrating the intimate relationship between DNA superhelicity and nucleosomal stability: When random single stranded breaks were introduced into the genomic DNA using a frequent cutter nickase enzyme, or by DNase I, the exogenous breaks significantly decreased nucleosomal binding of the H2A family members (H2A, H2A.X and H2A.Z) during salt elution, but not in intercalator elution. On the contrary, intercalator elution profiles were, salt elution profiles were not shifted upon nickase treatment in the case of H3.

The spectacular histone mobilizing effect of nicking treatments allowed us to determine the distance range of propagation of nucleosome destabilization along the supercoiled chromatin loops. The nicks, generated at nickase concentrations where nucleosome eviction already involves the majority of nucleosomes (0.05 U/ml), delimit ≥10 kb fragments, and this is the case even at 10x lower nickase concentrations.

B. 2. 4 General characteristics of the elution assays

To test wether the elution curves are independent of the expression levels of histones examined, nuclei of H2B-GFP expressing cells were gated according to the fluorescence intensity of GFP and elution curves were recorded in the nuclei showing low, medium and high GFP signals. The elution curves measured on nuclei with different H2B-GFP levels were similar to each other.

Reproducibility of the method from experiment-to-experiment was demonstrated by calculating the average and standard deviation of three independent measurements in the case of canonical H2B and H3 and also in the case of H3K4me3 and H3K27me3 modifications using salt elution or intercalator elution assays.

To asses that the elution profile of a particular histone may reflect the effects of its intra- or internucleosomal interactions, immuno-cross-linking experiments were performed in

the case of H2A.Z found to be unusually stable. The H3-like stability of H2A.Z containing dimers, mediated by its PWWP2A binding domain, may be the consequence of the interaction between H2A.Z C-terminal tail and the tetrasome which could be facilitated by increasing ionic strength. Nuclei were pretreated with different concentrations of salt solutions (in a range where H3 histones remain chromatin bound), then H2A.Z histones were X-linked by antibody labeling. Increasing amounts of H3 histones remained chromatin-bound as a result of immuno-X-linking of H2A.Z.

In contrast, the two members of the dimer within the nucleosomes was independetly dissociated from the chromatin measured in immuno-X-linking experiments using salt or inttercalator as nucleosome destabilizing agents.

B. 3. DISCUSSION

Our slide-based cytometric method offers a simple means to assess and compare stability features of nucleosomes consisting of native histones *in situ*. Its novelty stems in its combinative features, exploiting the advantages of cytometric analyzes in measurements based on exposure of chromatin to various destabilizing agents.

Our method is uniquely suitable for rapid screening of several features at the same time (e.g. to compare the stability of nucleosomes distinguished by different PTMs), or to determine the size of supercoiled loops in different PTM context (comparing H3K4me3 and H3K27me3 e.g., as it was done for bulk H3).

QINESIn demonstrated that phosphorylation on the C-terminus of H2A.X has a destabilizing effect on the nucleosome. Since the long stretches of chromatin packed with γH2A.X are generally visualized as being initiated at a double-strand break, the destabilized nature of γH2A.X nucleosomes may be due to these breaks, in line with the observations presented here.

QINESIn also demonstrated that H2A.Z containing dimers were unusually stable. Analyzes of a H2A.Z mutant lacking the C-terminal reader binding site has revealed that the unusually stable characteristics of H2A.Z containing nucleosomes are likely the consequence of the binding of the PWWP2A reader protein. This finding may lead to a novel paradigm regarding the role of histone variant composition and PTMs in transcriptional regulation with nucleosome destabilization taking central stage.

The dramatic effect of nicking on nucleosome stability suggests that regulation of superhelicity may be exploited by the cell for gene regulatory. Relaxation of superhelical

loops by nicking only once at ≥10 kb regions is apparently sufficient to destabilize most nucleosomes among our experimental conditions along the entire superhelical domain affected. The incidence of nicks necessary for nucleosome eviction likely overlaps the size of the supercoiled chromatin loops. Thus, local topological changes under physiological conditions, such as those arising from topoisomerase action, may also have important effects on the stability of a relatively large number of nucleosomes. It is tempting to speculate that such a loop-wide nucleosomal destabilization could be “read” by the cell at specific nucleosomes in a site-specific manner with the help of locally acting reader proteins or chromatin remodelers.

C. CYTOMETRIC MICROBEAD ASSAYS FOR EPIGENETIC ANALYZIS

C. 1. INTRODUCTION

We could make good use of our experience acquired in flow cytometric microbead analyzes to answer some of the questions related to the epigenetic research projects of our lab, studying the occurence of different PTMs on the same nucleosome. More than one histone tail can be modified within a particular nucleosome at the same time, and more than one amino acid residue can be modified on the same histone tail; the combinations of histone modifications are major determinants of epigenetic regulation.

Using a flow cytometric platform, I have demonstrated that detection of intranucleosomal combinations of epigenetic modifications is possible by capturing of mono-nucleosomes on microbeads via PTM-specific antibodies and subsequent analyzes of the bead-bound nucleosomes for other modifications, using immunofluorescence labeling.

I have also used this approach in the analyzes of endogenous or exogenous ss DNA breaks (nicks), and their specific chromatin environment (histones carrying certain post-translational modifications, RNA/DNA-hybrids (R-loops), epigenetic traits in the focus of interest in our lab.

Transient, endogenous single- or double-strand DNA breaks are generated in the nucleus of a living cell in a programmed manner in the context of physiological molecular mechanisms such as transcription. During transcriptional elongation, e.g., the movement and rotation of RNA Pol II along the DNA results in the generation of positive supercoils in front of the polymerase and negative supercoils behind it, thus torsional stress-resolving enzymes, topoisomerases, are essential components of transcription machinery. On the other hand, promoter-proximal, transient ds breaks accompany gene activation, relying on TOP2β.

R-loops are by-products of transcription, containing an RNA/DNA hybrid and a single-stranded DNA displaced by the RNA strand. The proposed physiological functions of R-loops involve class switch recombination of the immunoglobulin loci, protection of high GC content-promoters from DNA methylation and the facilitation of transcription termination at terminators of certain genes.

C. 2. RESULTS

C. 2. 1. Detection of PTM-specific histone associations

A microbead based cytometric method was developed to detect the co-occupancy of different histone PTMs on the same nucleosome or on the same histone molecule.

PTMs associated with endogenous γH2A.X histone variant were investigated. Streptavidinated microbeads were coated with biotinylated γH2A.X specific mouse antibodies and the chromatin was fragmented to 200-750 bp by sonication. The captured nucleosomes were labeled with H3K4me3 and H3K27ac specific primary antibodies and fluorophore-conjugated secondary antibody, and quantified by flow cytometer. It was shown that endogenous γH2A.X histone variants co-occur with H3K4me3 and with H3K27ac on the same nucleosome. The co-localization of H3K4me3 and γH2A.X was confirmed by superresolution STED microscopic analyzis.

C. 2. 2. Detection of nick-proximal RNA Pol II and R-loops

Based on the above, the polymerase based nick-labeling strategy was used to confirm our genomic data showing enrichment of endogenous nicks at active promoters of *Saccharomyces cerevisae* genomic DNA.

I performed a global „ChIP-on-beads" analyses of nick – RNAP II co-localization. Nickase was used to introduce random nicks to the samples that served as negative control exhibiting no RNA Pol II enrichment

In the nick-translated samples (NT) Ser5-P signals were higher than Ser2-P signals and this difference was eliminated if random nicks were generated by nickase before nick-translation.

I also tested the microbead-based cytometric approach to detect R-loops in the close proximity of endogeneous single-strand DNA discontinuities at the whole genome level, in an attempt to confirm and further analyze earlier observations of the group.

R-loops were detected in the vicinity of nicks with 3'OH, when biotin labeling was performed with Pol I.

C. 3. DISCUSSION

The above observations demonstrate that cytometric microbead assays can give an independent assessment of co-localizing epigenetic marks, what can be used in conjunction with, or as an alternative approach to, sequential ChIP (re-ChIP) procedures. The method was veryfied detecting co-occurence of endogenous γH2A.X either with H3K4me3 or H3K27ac confirming published data. The co-localization of γH2A.X and H3K4me3 was further confirmed by superresolution STED microscopy.

Applying biotin conjugated secondary antibodies, the method can be applied in a versatile manner. Using a panel of antibodies specific for histone PTMs or histone variants, this assay can serve as a feasible tool to perform simple, time- and cost-effective screening

studies for the evaluation of histone PTM and histone variant combinations present on nucleosomes.

The assay could also be used to investigate if endogenous nicks and R-loops on the one hand, and nicks and RNA Pol II species on the other are in molecular proximity. The presence of R-loops in the immunoprecipitates when DNA Pol I was used for nick-labeling extends our earlier data by suggesting proximity of the two entities within a nucleosomal distance. On the other hand, accumulation of the nick signal near sites of RNA Pol II Ser 5-P enrichmentdetected by the “ChIP-on-beads” approach in the *S. cerevisiae* genome is in line with the possibility that the ss breaks appear in concert with RNAP II-related transcriptional activity.

These observations illustrate the utility of the “ChIP-on-beads” approaches described in epigenetic research. The flow and laser scanning cytometric platforms could be interchangeably applied in the assays demonstrated.

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION

In view of the data presented here, flow cytometry can be utilized for the detection of local genetic alterations. Introduction of this strategy for routine diagnostic purposes may be facilitated by the readily available instrumentation and the wealth of relevant experience already present in many laboratories. It may be offered in the case of demand for high-throughput multiplex assays for the simultaneous analyzes of a limited number of genetic conditions in large populations of human and non-human biological samples. The assay based on the differential melting of PCR products of different length was patented in the Hungarian Intellectual Property Office as „Cytometric process for comparative analyzis of PCR products lengths and the use of this process".

Quantitative microscopy has proved useful in the epigenetics field as a tool of global analyzes; the analytical platform described here greatly extends its utility yielding data of direct functional relevance. QINESIn is a very promising approach with high-throughput screening potential what could be exploited e.g. for the chemical profiling of the genome in search of intercalators with PTM, or histone variant specific effects.

The peculiar stability of H2A.Z containing nucleosomes presented herein provide a good example for the utility of QINESIn in assessing nucleosome stability features affected by reader proteins.

The assay was registered in the Hungarian Intellectual Property Office as „Quantitative *in situ* measurement of histone-DNA interaction.".

It was also demonstrated that flow and laser scanning cytometric analyzes can be extended to the field of epigenetics. ChIP combined with MMA (multiplex microbead assay) could be a relevant alternative of Western blot or re-ChIP techniques offering multiplex high-throughput analyzes of histone PTM or histone variant combinations of nucleosomes.

CONCLUSIONS:

1. Methodical. The array of methods elaborated greatly extend the methodical possibilities of cytometry, could serve as the bases for the development of HTP tests of genetic and epigenetic alterations and constitute a versatile platform for investigations, realizing the concept of a „lab-on-beads".

2. Biological. A number of conclusions of biological relevance have been reached that are listed below to illustrate the wide spectrum of problems that can be efficiently addressed using these approaches:
    2.1. The relatively destabilized nature of H3K4me3 modified nucleosomes is a general feature, independent of cell type and differentiation state, and it is limited to the promoter-proximal nucleosomes.
    2.2. Relaxation of superhelicity by nicks have a major effect on nucleosome stability.
    2.3. The destabilizing effect of nicks spreads to loop size distances.
    2.4. Phosphorylation decreases the intrinsic stability of dimers containing histone variant H2A.X.
    2.5. The majority of H2A.Z containing dimers are unusually stably associated with the tetrasomes.
    2.6. Stability of H2A.Z containing dimers does not not depend on the isotypes of H2A.Z
    2.7. H2A.Z containing dimers comprise an intercalator resistant subpopulation.
    2.8. The unusually salt- and intercalator-resistant characteristics of H2A.Z are due to the binding of the PWWP2A reader protein.
3. A number of new questions of biological significance have been raised in the wake of our studies:
    3.1. Are there any other PTMs that affect nucleosome stability?
    3.2. Does the destabilizing role of PWWP2A represent an example of a paradigm for other other histone variants and perhaps for PTM sas well?
    3.3. What is the exact relationship between free 3’OH groups of R-loop origin and those defining bona fide endogenous nicks?

MATERIALS AND METHODS

Chemicals, DNA isolation, plasmids

All reagents were from Sigma-Aldrich (St. Louis, Missouri, USA) unless otherwise stated. Genomic and plasmid DNA were prepared by the Intron G-DEX Genomic DNA Extraction Kit and DNA-midi Plasmid DNA Purification Kit (Intron Biotechnology, Seongnam-Si, South Korea) following the manufacturer's instructions. The plasmids carrying different length CAG repeats or different isoforms of the histone variant H2A.Z were transformed into *Escherichia coli* DH5α by heat shock and selected on LB plates containing 100 μg/ml ampicillin.

Cells and clinical samples

HeLa cells expressing H2B-GFP, H3-GFP and H4-GFP fusion proteins, and HCT116 cells (Developmental Therapeutics Branch, National Cancer Institute, Bethesda, Bethesda, MD, 20892) were cultured in DMEM supplemented with 10% FCS, 2 mM l-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin. Jurkat cells were cultured in RPMI1640 medium supplemented with 10% FCS, 2 mM l-glutamine 100 μg/ml streptomycin, 100 U/ml penicillin, Wild type, H2A.Z1 knock-out, H2A.Z2 knock-out, double knock-out and mutant H2A.Z1 expressing DT-40 chicken B cells (provided by Masahiko Harata) were cultured in DMEM supplemented with 2% chicken serum, 8% FCS, 2 mM l-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin.

Genomic DNA derived from anonymous patients diagnosed with Huntington's disease, confirmed by sequence analyzes and polyacrylamide gel electrophoresis using two different sets of primers, were isolated at the Department of Medical Genetics, School of Medicine, University of Pécs. Genomic DNA with BRCA1 5382 insC mutation was from the Department of Clinical Biochemistry and Molecular Pathology, Medical and Health Science Center, University of Debrecen. Genomic DNA derived from peripheral blood lymphocytes of healthy volunteers were isolated in our laboratory.

PCR reactions

<u>Huntington's disease:</u>

The primers used for amplification of the CAG repeats in the exon 1 of the Huntingtin gene (IT15/HTT/HD) were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Forward primer, Hunt1: biotin-5'-CAT GGC GAC CCT GGA AAA GCT G-3', reverse

primer, Hunt2: Cy5-5’-GGC GGT GGC GGC TGT TGC TGC TGC TGC TG-3’ and reverse primer, Hunt3: Cy3-5’-GGC GGT GGC GGC TGT TGC TGC TGC TGC TG-3’. The PCR reactions were performed as described in ref. (Muglia et al. 1996).

5382 insC point mutation BRCA1:

The wild type allele specific forward primer (BRCA1: Cy5-5'-AAA GCG AGC AAG AGA ATC GCA-3'), the mutation specific forward primer (BRCA2: Cy5-5'-CAG CAG CAG CAG CAG CAG CAC CTT AGC GAG CAA GAG AAT CAC C-3', containing a 5’ tag of a 18 bp CAG repeat) and the common reverse primer (BRCA3: biotin-5'-AGT CTT ACA AAA TGA AGC GGC CC-3') were used together in a multiplex reaction. The primers determine a 126 bp long fragment of the human BRCA1 gene, including the site of the 5382 insC point mutation.

Heteroduplex analyzis:

The template was the pMEP4 plasmid DNA containing the breakpoint cluster region of the human MLL gene (from Peter Aplan, NIH, Bethesda), throughout the experiments. All primers (pIL1 forward 5’–ATA TGA ATA CTC ATC ACT GAG TGC CTT TGG C–3’; pIL1-biotin forward 5’–(biotin)-ATA TGA ATA CTC ATC ACT GAG TGC CTT TGG C–3’; pM3 forward 5’–GCT GGA GTG TAA TAA GTG CCG A–3’; pM6 reverse 5’–AGC GAA CAC ACT TGGTAC AGATC–3’; pM6-6FAM reverse 5’–(6FAM)-AGC GAA CAC ACT TGG TAC AGA TC–3’) were purchased from Integrated DNA Technologies (Coralville, IA).

Preparation of single-stranded DNA by linear amplification:

Amplification was performed using either the pIL1-biotin (sense) or the pM6-6FAM (antisense) primer alone. Unlabeled ds PCR products of 574 or 357 bp length were used as template DNA in these experiments.

Hybridization of PCR products and cleavage by Pvu II

The longer 6FAM- and, in a separate test tube, the shorter 6FAM-labeled linear amplicons were mixed with equal amounts of the biotin labeled linear longer product, in TE supplemented with 1 M NaCl. The two samples were denatured at 95ºC for 5 min, then annelaled at room temperature (RT) for 2 h.

Two hundred nanograms of the hybrids formed between the 6FAM- and biotin labeled PCR products were digested with 10 U Pvu II restriction endonuclease (Fermentas, Life Sciences).

Binding of PCR products to beads

Biotinylated and fluorescently labeled PCR products were added to 10 000 polymeric streptavidin coated beads (6 μm diameter, purchased from Polysciences Inc., Warrington, Pennsylvania, USA) in 200 μl 1×PBS (150 mM NaCl, 3.3 mM KCl, 8.6 mM Na_2HPO_4, 1.69 mM KH_2PO_4, pH 7.4) and incubated at RT for 40 min in the dark. After incubation, the beads were washed and centrifuged at 20 000 g for 10 min.

Heat treatment in formamide

The beads carrying PCR products on their surface were treated with a concentration series of formamide diluted in ddH_2O. In the experiments related to Huntington's disease the concentration of formamide was titrated between 65-75 v/v % to find the optimal concentration where the PCR products with different length can be distinguished. In the case of the BRCA1 5382insC point mutation, formamide was titrated between 57-62 v/v %. Heat treatment was performed in a total volume of 200 μl, in a PCR tube containing approximately 10 000 beads. The beads were incubated with formamide at 40°C in the dark for 3 min, washed and analyzed by flow cytometry

Coating of beads by biotinylated anti-γH2A.X antibody

10 μg of the biotinylated mouse monoclonal anti-γH2A.X (Merck-Millipore, Darmstadt, Germany; 1 mg/ml) antibody was immobilized on the surface of 0.1 mg streptavidinated magnetic Dynabead (Thermo Fisher Scientific, Waltham, Massachusetts, USA), by rotating overnight at 4°C.

Detection of combinatorial histone PTMs

Jurkat cells were washed then fixed in 5 ml 1% formaldehyde. After fixation, formaldehyde was quenched by 2.5 M glycine. Cells were washed and resuspended in IP buffer (1% SDS, 50 mM Tris-HCl pH 8, 10 mM EDTA, 1mM PMSF). Lysis was achieved by pipetting the cells up and down several times. The cell lysates were centrifugated (12 000 g, at 4ºC), and the pellet was resuspended in IP buffer and sonicated (Bioruptor, Diagenode, New Jersey, USA). The sonicated samples were centrifugated and resuspended in ice-cold IP buffer. The sonicated samples were divided to aliquots equivalent of 10^7 cells. 500,000 Dynabeads previously coated with anti-γH2A.X antibody were added to the sonicated samples and incubated overnight at 4ºC on a rotator. The beads were washed and the nucleosomes immobilized on the beads were labeled with rabbit polyclonal anti-H2A.X (Abcam, Cambridge, UK; 1 mg/ml), rabbit polyclonal anti-H3K4me3 (Abcam, Cambridge, UK; 1 mg/ml) or rabbit polyclonal anti-H3K27ac (Abcam, Cambridge, UK; 1 mg/ml)

primary antibody then with fluorophore-conjugated secondary antibody supplemented with propidium-iodide.

Detection of nicks and nick proximal R-loops

Human peripheral blood lymphocyte (hPBL) cells were isolated by Ficol gradient centrifugation. The cells were washed with PBS and fixed with 1% formaldehyde. An equal volume of cell suspension and 1% LMP diluted in 1×PBS, both kept at 37°C, were mixed, resulting in a 0.5% final concentration of LMP. Agarose blocks containing agarose/cell suspension were washed andand cells were lysed in lysis buffer (1% Sarcosyl, 10 mM Tris-HCl pH 8, 0.4 M EDTA, 0.5 mg/ml Proteinase K) at 55°C for 24 hours. The lysed cells were washed with TE (10mM Tris, 1 mM EDTA pH 8.0) and treated with PMSF. The blocks were equilibrated with Pol I or with TdT bufferand were nick-translated by DNA Pol I (1×DNA Pol I buffer, 5 µM ddNTP mix, 1 µM dNTP mix, 22.5 U/block DNA Pol I enzyme) or by TdT (1×TdT buffer, 5 µM biotin-dUTP, 10 U/block TdT enzyme). Reaction was stopped and blocks were washed with TE. Blocks were melted and the agarose was digested by β-agarase enzyme. Digested blocks were sonicated, DNA was cleaned and immobilized on streptavidinated Dynabeads. After immobilization, the beads were washed and labeled with the S9.6 RNA/DNA-hybrid specific antibody. Beads were washed and labeled with fluorophore conjugated secondary antibody supplemented with propidium-iodide. After antibody labeling, the beads were washed and measured by a flow cytometer.

Flow cytometry

Melting point analyzes_were performed using a Becton Dickinson FACSArray instrument (San Jose, California, USA). Fluorescence signals were detected in the PI/Yellow and Far Red channels through the 585/42 and 675 LP interference filters of the instrument, respectively.

Heteroduplex analyzes were performed using a Becton Dickinson FACScan instrument (San Jose, California, USA). Fluorescence signals were detected through the 530/30 and the 585/42 interference filter of the instrument, designated as FL1 and FL2 channels, respectively.

Detection of combinatorial histone PTMs and nick proximal R-loops was performed using a Becton Dickinson FACS Aria III instrument (San Jose, California, USA). Fluorescence signals were detected in the PI/Yellow and Far Red channels through the 585/42 and 675 LP interference filters of the instrument, respectively.

FACS Aria III measurements were performed by Szabolcs Tarapcsák.

Global „ChIP-on-beads" assay

Preparation of nuclei:

The pellet of *S. cerevisae* cells was resuspended and fixed in 4% paraformaldehyde. After quenching in 2.5 M glycine and washing twice with 50 mM Tris-HCl (pH 7.5)/30 mM DTT, the cells were treated with lyticase. The spheroplasts were washed, lysed in buffer N (30 mM HEPES pH 7.6, 3 mM DTT, 25 mM Na_2SO_4, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 7.2 mM spermin, 1 mM PMSF) and disintegrated using a Dounce homogenizer.

Nickase treatment and nick translation:

The nuclei were washed in 1×PBS/EDTA followed by washing in nickase buffer (10 mM Tris-HCl pH 8, 50 mM NaCl, 10 mM $MgCl_2$, 1 mg/ml BSA). Random nicks were introduced into the DNA of control samples using the frequent cutter Nt.CviPII nickase enzyme (recognition site: CCD; New England Biolabs Inc., Ipswich, Massachusetts, USA). Limited nick translation of the samples harboring only endogenous nicks and of the nickase treated control was performed as described above.

Fragmentation of chromatin, microbead capture:

The nick-translated samples of nuclei were disintegrated using a FastPrep-24™ 5G Instrument bead beater (MP Biomedicals, Santa Anna, California, USA) and sonicated with a Bioruptor Plus sonicator (Diagenode, Denville, New Jersey, USA). Streptavidinated Dynabeads (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were added to the sonicated. The beads were washed and embedded into agarose in an 8-well chamber (Ibidi, Martinsried, Germany) see below.

Immunofluorescence labeling:

The agarose embedded Dynabeads were washed with lysis buffer supplemented with 360 mM NaCl, with washing buffer (10 mM Tris-HCl pH 8, 250 mM LiCl, 0.5% Na-deoxycholate,1mM EDTA), finally with 1×PBS/EDTA. Indirect immunofluorescence labeling was performed using rabbit polyclonal anti-RNAP II Ser2-P (Abcam, Cambridge, UK; 1 mg/ml) or rabbit polyclonal anti-RNAP II Ser5-P (Abcam, Cambridge, UK; 1 mg/ml). After labeling with the primary antibodies, the beads were washed and labeled with the secondary antibody. After labeling, the agarose embedded beads were washed, stained with propidium–iodide and fluorescence intensity distributions were recorded using a laser scanning cytometer (LSC; see below).

Determination of background fluorescence:

Following the first LSC measurement, the agarose embedded Dynabead samples were equilibrated with DNase I buffer (10 mM Tris-HCl pH 8, 0.1 mM $CaCl_2$, 2.5 mM $MgCl_2$) and digested with DNase I. After enzymatic treatment, the agarose-embedded beads were washed and measured again by LSC in a second run, performed on the same beads. The mean fluorescence intensity remaining after DNAse digestion was subtracted from the mean of the first LSC run, to correct for the background fluorescence.

Embedding live cells into low melting point agarose

Prior to embedding the wells of 8-well chambers (Ibidi, Martinsried, Germany) were coated with 1% (m/v) low melting point (LMP) agarose.

The cell suspension was mixed with 1% LMP agarose, dispensed in the middle of the wells and covered with home-made rectangular plastic coverslips.

Preparation of nuclei/permeabilization and histone eviction by salt or intercalators

After polymerization of the agarose the embedded cells were permeabilized with 1% (v/v) Triton X-100. After permeabilization, nuclei were washed and were treated with different concentrations of NaCl or intercalator solutions.

Immunofluorescence labeling

After salt or intercalator treatment the samples were blocked with Blotto Non-Fat Dry Milk (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). The blocking solution was washed out and indirect immunofluorescence labeling was performed using rabbit polyclonal anti-H2A (Abcam, Cambridge, UK; 0.4 mg/ml), rabbit polyclonal anti-H2A.X (Abcam, Cambridge, UK; 1 mg/ml), mouse monoclonal anti-γH2A.X (Merck-Millipore, Darmstadt, Germany; 1 mg/ml), mouse monoclonal anti-H3K4me3 (0,5 mg/ml) or mouse monoclonal anti-H3K27me3 (0,5 mg/ml) primary antibodies. After labeling with the primary antibodies, the nuclei were washed and labeled with the secondary antibodies. Then the samples were fixed in 1% formaldehyde and were stained with propidium–iodide. The stained nuclei were washed and fluorescence intensity distributions were recorded using an iCys laser scanning cytometer (LSC), as described below.

Etoposide treatment

Agarose embedded live cells were treated with etoposide (TEVA, Debrecen, Hungary). The drug was diluted in complete DMEM medium and the cells were incubated together with the drug at 37°C in 5% CO_2 atmosphere.

Nickase and DNase I treatment

Live cells were embedded into agarose as described above and treated with lysis buffer (0.4% (v/v) Triton X-100, 300 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH8, followed by treatment with 1% (v/v) Triton X-100. The frequent cutter Nt.CviPII nickase (recognition site: CCD; New England Biolabs Inc., Ipswich, Massachusetts, USA) and DNase I were applied after the washing steps following permeabilization (see above).

Immuno- cross-linking experiments

Agarose embedded and permeabilized HeLa cells expressing H2B-GFP were blocked and indirect immunofluorescence labeling was performed using rabbit polyclonal anti-H2A antibody. After labeling with the anti-H2A antibody, the nuclei were washed and anti-H2A labeled histones were cross-linked with the secondary antibody using A647-conjugated goat anti-rabbit IgG.. After labeling with the secondary antibody, nuclei were washed and the samples were treated with NaCl or doxorubicin solutions as described above. Then the samples were fixed with formaldehyde, stained with propidium iodide and the fluorescence intensity distributions were recorded by LSC, as described above.

Gel electrophoretic analyzes

The permeabilized, agarose-embedded H2B-GFP expressing samples were deproteinized as described earlier. Standard, nondenaturing agarose gel electrophoresis was perfomed with or without post-treatment of the nickase digested samples with S1 nuclease (1000 U/ml) used in its own buffer (1h at 37°C) to convert nicks into double-strand breaks. For pulsed-field gelectrophoresis of the S1-treated samples, either a CHEF mapper XA Pulse Field Electrophoresis System (Bio-Rad Laboratories Inc., Hercules, California, USA) was used following the manufacturer's instructions to resolve 3-300 kb fragments, or an MJ Research PPI-200 power inverter (Field Inversion Gelectrophoresis; FIGE) running 'program 5' to resolve the same size range. For markers, O'GeneRuler 1kb DNA ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA), lambda DNA (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Pulse Marker 2200-225 kb were used.

Automated microscopy

Automated microscopic imaging was performed using an iCys instrument (iCys® Research Imaging Cytometer; CompuCyte, Westwood, Massachusetts, USA). Green fluorescent protein (GFP), SYBR Gold, A488, doxorubicin and PI were excited using a 488 nm Argon ion laser, A647 with a 633 nm HeNe laser. In the case of elution assays fluorescence signals were collected via an UPlan FI 20× (NA 0.5) objective, scanning each

field with a step size of 1.5 µm. For the measurment of agarose embedded microbeads, fluorescence signals were collected via an UPlan FI 40× (NA 0.65) objective, scanning each field with a step size of 0.5 µm. GFP and A488 were detected through 510/21 nm and 530/30 nm filters, respectively, while doxorubicin, A647 and PI were detected through a 650/LP nm filter.

Confocal Laser Scanning Microscopy (CLSM)

Confocal images were taken using an FLUOVIEW FV 1000 confocal microscope (Olympus, Center Valley, Pennsylvania, USA) based on an inverted IX-81 stand with an UPLS APO 60× (NA 1.35) oil immersion objective. GFP or A488 were excited by a 488 nm Argon ion laser. A647 and PI were excited by a 633 nm and 543 nm HeNe laser. Image analyzis was performed using the Image J software (http://imagej.nih.gov/ij/).

STED microscopy

Superresolution images were taken by a Nikon NiE (Upright) microscope using a 100x NA=1.45 objective. For immunofluorescence labeling, antibodies conjugated with fluorophores (Abberior star 580, Abberior star red) appropriate for STED imaging were used as secondary antibodies. Co-localization was calculated by Image J software. STED microscopy was done by Péter Nánási.

Chromatin immunoprecipitation, sequencing, bioinformatics analyzes

Chromatin immunoprecipitation, sequencing (ChIP-Seq) and ChIP-qPCR experiments were carried out as in (Barish et al. 2012), with minor modifications. ChIP-Seq libraries were prepared from two biological replicates by Illumina according to manufacturer's instructions. ChIP-Seq experiments were performed by Zoltán Simándi.

Primary analyzes of the ChIP-Seq raw reads was carried out using a ChIP-Seq analyze command line pipeline. Control and doxorubicin-treated H3K4me3 samples were analyzed by DiffBind v1.0.9 (with parameters 'minOverlap = 2' and 'full library size'), using duplicates. Integrative Genomics Viewer (IGV 2.3, Broad Institute) was used for data browsing. Normalized tag counts for Meta histogram and Read Distribution (RD) plots were generated by annotatePeaks.pl with options '-ghist' and '-hist 25' from HOMER and then visualized by R using or Java TreeViewer. Bioinformatic analyzes were performed by Attila Horváth.

Sample preparation for MS measurement

H3-GFP expressing HeLa cells were embedded into agarose blocks. Blocks were washed and permeabilized with 1% Triton-X 100. After washing blocks were treated with

EBr. The proteins that remained in the nuclei after EBr treatment were eluted with 2 M NaCl. Eluted proteins were concentrated in a 10K Amicon tube (Merck-Millipore, Darmstadt, Germany) and the buffer was changed to 1×PBS. Proteins eluted from the filter were stored at -20°C for MS analyzis.

SDS-PAGE and LC-MS/MS analyzes

Proteins were analyzed on a 10% SDS polyacrylamide gel. Electrophoresis was carried out in a Bio-Rad mini tetra cell (Bio-Rad).

SDS-PAGE analyzes were done by Gergő Kalló at the Proteomics Core Facility of the Department of Biochemistry and Molecular Biology, University of Debrecen.

The bands of SDS-PAGE were excised, followed by in-gel digestion with trypsin. Reduction was performed using dithiothreitol, followed by alkylation. Trypsin digestion was performed and the digested peptides were extracted and lyophilized. The peptides were re-dissolved in formic acid before mass spectrometric analyzis. The acquired LC-MS/MS data were used for protein identification with the help of Protein-Pilot 4.0 (ABSciex) search engine and the SwissProt database, using the biological modification table included in the ProteinPilot 4.0 software. A minimum of two peptide sequences with 95% confidence were used for protein identification.

MS analyzes were done by Gergő Kalló at the Proteomics Core Facility of the Department of Biochemistry and Molecular Biology, University of Debrecen

SUMMARY

Novel cytometric methods were developed for genetic and epigenetic analyzes: (A) for the detection of certain genetic alterations of the length or in the sequence of short genomic regions, (B) for the *in situ* evaluation of nucleosome stability and (C) for the "ChIP-on-beads" analyzes of nucleosome-associated epigenetic modifications.

(A) The flow cytometry assay to address genetic diseases is based on the melting point analyzis of PCR products carrying biotin and a fluorescent moiety on their two ends, and are immobilized on streptavidin-coated microbeads. The efficacy and sensitivity of the method is demonstrated in the case of CAG triplet expansion in Huntington's disease and a BRCA1 point mutation.

(B) The laser scanning cytometric assay for the purposes of *in situ* evaluation of nucleosome stability is based on the elution of histones using intercalators or salt, so as to assess stability features dependent on DNA superhelicity or electrostatic interactions, respectively. The assays can be preformed on a PTM or histone variant dependent manner. The utility of the method is demonstrated via the comparative analyzes of different histone PTMs and histone variants, including H2A.Z, H2A.X and its phosphorylated form, γH2A.X. It was also demonstrated that DNA relaxation (by nicking) destabilizes nucleosomes, what underscores the powerful potential of topological relaxation in the epigenetic regulation of DNA accessibility. Notably, nicking at ≥10 kb intervals is sufficient to induce global nucleosome destabilization, demonstrating that relaxation is propagated along most of the nucleosomes of the relaxed chromatin loops. The novel observations of biological significance I made employing this method include the demonstration of the nucleosome stabilizing effect of a reader protein in the case of H2A.Z.

(C) The cytometric assays for the "ChIP-on-beads" analyzes of nucleosome-associated epigenetic modifications are based on the measurement of PTM combinations on isolated mononucleosomes immobilized on microbeads. This experimental system proved useful also for the differential detection of initiating and elongating RNA Pol II molecules and R-loops juxtaposed with nicks.

As a general, methodical conclusion, suitable combinations of cytometric approaches with molecular biological methodology can offer a novel outlook on a wide variety of genetic and epigenetic questions of both practical and fundamental biological importance, with many potentialities yet to be exploited.

PUBLICATIONS

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Candidate: László Imre
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Doctoral School: Doctoral School of Molecular Cellular and Immune Biology
MTMT ID: 10038939

List of publications related to the dissertation

1. Hegedűs, É., Kókai, E., Nánási, P. P., **Imre, L.**, Halász, L., Jossé, R., Antunovics, Z., Webb, M. R., El Hage, A., Pommier, Y., Székvölgyi, L., Dombrádi, V., Szabó, G.: Endogenous single-strand DNA breaks at RNA polymerase II promoters in Saccharomyces cerevisiae.
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