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Title	Analysis of chromatin structure and dynamics with single nucleosome imaging
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Analysis of chromatin structure and dynamics with single nucleosome imaging

Tadasu Nozaki

Abstract

DNA is the media of hereditary information of living organisms on the earth. In eukaryotes, long genomic DNA is wrapped around core histones to form a nucleosome fiber. In addition, nucleosome fiber is organized three dimensionally as chromatin and folded within a tiny space such as a nucleus or chromosomes. In cellular functions based on DNA transaction including transcription and DNA replication, the cells must precisely execute the readout of information superimposed on genomic DNA. In this situation, a protein as molecular machinery needs to move around such complicated folded DNA and interrogate its target site, and the chromatin becomes a huge obstacle. Thus, chromatin structure and dynamics seem to be deeply related to diverse cellular functions but not merely packaging, and play a fundamental role in epigenetic regulation. Therefore, making the detailed observation and description of chromatin is essential to understand the various life phenomena in eukaryotes. The objective of this thesis is to contemplate the relationship between chromatin and epigenetics. To achieve this objective, multiple methods including genome-wide analysis of epigenetic regulation and live cell imaging of chromatin were performed. First, the epigenetic regulation and promoter types were analyzed based on genome-wide ChIP-Seq and DeepCAGE data. It was found that, in humans, broad promoters but not peak promoters had significant associations with histone modification and nucleosome position. Secondly, single nucleosome imaging was newly developed. To observe single nucleosomes in living mammalian cells, the histone was fused with photoactivatable (PA)-green fluorescent protein (GFP) and expressed in mammalian cells at a very low level. For single nucleosome imaging, an oblique illumination was used to illuminate a limited thin area within the cell. It was found that a small fraction of histone-PA-GFP was spontaneously activated without laser stimulation. Lastly, based on single nucleosome imaging, the chromatin structure and dynamics at single nucleosome level in a living mammalian cell and their variation related to different types of histone modification and chromatin associated proteins were successfully observed. Furthermore, numerous chromatin domains were identified throughout the cell cycle. These results will provide a basis of dynamic and flexible nature of chromatin toward understanding the function of chromatin in eukaryotic cell and allow new insight and perspective on the relationships between chromatin and epigenetics.

Keywords: chromatin, mitotic chromosome, epigenetics, single molecule imaging, transcriptional regulation

In this digest, I show you a part of chapter 1, the full part of chapter 3, the discussion part of chapter 4, and chapter 5 in my Doctoral dissertation.

1. Chapter1: Introduction

1.1. DNA as information media of cell

DNA is the media of hereditary information for living organisms on the earth. DNA consists of 4 types of nucleotides, A (adenine), T (thymine), G (guanine), and C (cytosine) and can make huge variation and satisfy the complexity for gene function. The gene is the unit of cell function and genome that is assembled of genes is the totality of information to characterize the species. Living organisms are created through expression process of genes. The gene coded on DNA is transcribed into the RNA and RNA is translated to the protein. Through such information processing, information of gene coded on DNA is translated and physically transformed into a protein, which acts as a molecular machinery in a cell. This information processing from DNA to proteins is named "central dogma" and living organisms on the earth go into this rule (Crick, 1970). Furthermore, to proliferate the number of cells and duplicate the genomic information, DNA is replicated and the genomic information is precisely transmitted from a cell to daughter cells at cell division. Additionally, DNA is stable to thermal perturbation and DNA replication keeps low error rate because of double helix, complementary strands, and DNA repair system.

Focusing on the cell, which is the basic unit of life, in macro-scale, we can find cells organized into a tissue, tissues into an organ, organs into an organism (Simon, 1991). Focusing on the cell in micro-scale, we can find the nucleus, cell membrane, cytoskeleton, endoplasmic reticulum, mitochondria on as the functional subcomponents. Each cell is comprised of subsystems including metabolic pathway, transcriptional system, DNA replication system, DNA repair system, immune system, signaling network, and so on. Like this, a living organism is comprised at hierarchical and complicated systems (Simon, 1991). The all information for creating and maintaining living organisms is certainly coded on DNA.

Furthermore, DNA is not electronic and digital but physical, therefore it has the three-dimensional entity including shape and length. In the eukaryote, the huge length of linear genomic DNA is packed into a tiny space. For instance, the total length of genomic DNA in a human diploid cell reaches approximately 2 m, but the diameter of the nucleus into which DNA is packed is just about 10 μm (a volume of only ~100 fL to 1 pL). Folding of DNA is complicated because of its negative charges with the phosphate groups and repulsion derived on them. Such a complicated folding of DNA is achieved by specialized proteins, which neutralize the negative charges of DNA, and the complex of DNA and binding proteins is called "chromatin". Nevertheless the detail of the folding process has been unclear (Figure 1.1).

To execute the information superimposed on DNA, proteins, for instance, transcriptional machinery, must interrogate their target sites on such complicated folded DNA. But how is it possbile? We know that a diploid cell has only two loci of the target gene and a small number of the transcription factor, which does not have "the map of nucleus leading to the target", have to find their target sites in limited time and with less energy. In our life, we usually use many efficient algorithms, for example, SSEARCH based on Smith-Waterman algorithm to perform pair-wise alignment of DNA sequences (Smith and Waterman, 1981a, b) and Google based on Page Rank algorithm to search information in the vast and infinite internet (Brin and Page, 1998), but how does the cell efficiently search and find the information on DNA? We are not even sure "the algorithm in cell" at all. Proteins in the nucleus search their target site by Brownian motion. When protein searches its target, chromatin structure becomes a big obstacle (Bancaud et al., 2009; Gorisch et al., 2005), so chromatin structure seems deeply related to diverse cellular functions including transcriptional regulation, DNA replication, DNA repair, and DNA recombination, but not merely packaging. Furthermore, the variation of readout of genetic information from identical genomes in a multi-cellular organism has been considered to be controlled by chromatin. Thus, for investigation of the search algorithm in a cell, making the detail observation and description of chromatin are required.

Although study of chromatin began in 19th century and chromatin is one of the oldest research subjects in cell biology, we have already understood only just a part of the chromatin structure

and function. Understanding the detail of chromatin leads us to understand “the algorithm in cell” and various cellular functions.

1.2. Objective

Described above, chromatin does not consist of just only DNA but also complex of nucleosomes and their binding proteins, and have important roles in epigenetics. The objective of this thesis is to reveal the relationships between chromatin and epigenetics. To achieve this objective, we connected multiple methods including genome-wide analysis and live cell imaging. Then, I set the aim of study in this paper; to reveal the relationships between epigenetic regulation and promoter types by bioinformatics approach and to reveal the chromatin structure and dynamics at nano-scale by the newly developed imaging method.

Previously, we could not detect the genome-wide histone modifications superimposed on chromatin, but now recent progress in high-throughput technologies has made it possible to collect a variety of “omics” data on transcripts and on the epigenetic behaviors of the histone modifications. ChIP-Seq method has been developed in analyzing genome-wide protein biding pattern including specific histone modifications. Currently, many sets of data of histone modifications and gene expression patterns are available. We focused on the relationships between epigenetic regulation based on histone modifications and promoter types, we connected ChIP-Seq data analysis to promoter analysis. This analysis provided a key to understanding epigenetic regulation in eukaryote (Chapter 2).

The range of observable world is determined by the power of microscopy. Due to the light diffraction limit, it was impossible to observe 10-200 nm ranged objects even though the size of chromatin domain is in 10-200 nm. Although this resolution of microscopy impedes the progress of chromatin research, newly developed single molecule imaging and super-resolution imaging make us possible to smash the light diffraction limit and observe the detail of chromatin. Chromatin environment: chromatin structure, dynamics and histone modifications are crucial components that are involved in the epigenetic regulation and thus studying chromatin environment will provide the new information towards understanding the various cellular systems in eukaryotes. In this manuscript, to observe the chromatin below the diffraction limit in a living cell, we developed the single nucleosome imaging method, which enables us to observe chromatin at single molecule level (Chapter 3). We further applied this single nucleosome imaging to super-resolution imaging and chromatin dynamics analysis overall nucleus. Super-resolution imaging reveals the formation of numerous chromatin domains in a living human nucleus and mitotic chromosome and demonstrated the formation system of the chromatin domains. Furthermore, base on single nucleosome imaging, we succeeded in measuring chromatin dynamics at single nucleosome level and creating the heatmap of dynamics of chromatin domain. This analysis shows the difference of chromatin dynamics at local region in nucleus and reveals the control system of chromatin dynamics. Finally, after we changed histone modification patterns or depleted chromatin associated proteins, we observed the change of chromatin environment (Chapter 4). I hope that our results will contribute to investigating epigenetics.

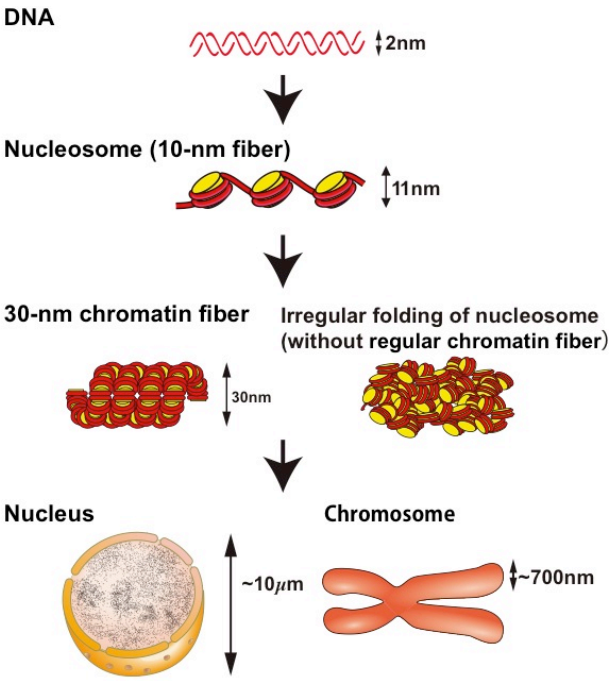

Figure 1.1 From DNA to chromatin
In eukaryotes, long genomic DNA is packed into a tiny space of nucleus or chromosomes. DNA whose diameter is 2 nm is wrapped around histone proteins comprised at 2 copies of each H2A, H2B, H3, and H4 protein and forms nucleosome. Histone proteins have positively charged amino acids containing the lysine and arginine, and they neutralize a part of negative charges on DNA. Nucleosomes have been assumed to be folded into regular 30-nm chromatin fiber. However, currently, nucleosomes have been considered to be folded irregularly and form chromatin domains that have not the regular size (figure from (Maeshima et al., 2014)).

2. Chapter 3: Single nucleosome imaging in living mammalian cells

2.1. Introduction

Approximately 2 m of human genomic DNA is organized in the cell. DNA is wrapped around core histones and forms a nucleosome fiber known as "beads-on-a-string." The nucleosome fiber has long been assumed to be folded into a regular 30-nm chromatin fiber and larger fibers folded into helical structures. However, several lines of evidence, including recent studies using cryo-electron microscopy and synchrotron X-ray scattering analyses (Eltsov et al., 2008; Joti et al., 2012; Nishino et al., 2012) demonstrated no regular structure >11 nm in interphase chromatin and mitotic chromosomes. These findings suggest that interphase chromatin and mitotic chromosomes consist of compact and irregularly folded nucleosome fibers without a 30-nm chromatin structure (i.e., a polymer melt-like structure) (Eltsov et al., 2008; Maeshima et al., 2010). This structure implies a less physically constrained and more locally dynamic state. Nucleosome fibers may thus be constantly moving and rearranging at the local level, which may be crucial for protein factors in the search for genomic DNA. How can we observe such local behavior of the nucleosome in living human cells? The obvious question was whether nucleosome fluctuation could be detected in living mammalian cells. For this purpose, we newly developed and performed single particle imaging of nucleosomes in living cells.

2.2. Materials and methods

2.2.1. Microscopy setup

Nikon laser TIRF microscope system Ti was used to observe the distribution of single H4-PA-GFP molecules expressed in DM (Indian Muntjac) cells. 488-nm laser (100 mW and ND 50 %) was introduced into the microscope through an optical path installed on a vibration insulation table. Through an objective lens (100x PlanApo TIRF, NA 1.49; Nikon), DM cells grown on a glass base dish (Iwaki) were exposed to the excitation light. The incident angle of the laser beam to the specimen plane was chosen so as to obtain a oblique illumination (Tokunaga et al., 2008). Collected fluorescence from the cells was focused on the sCMOS ORCA-Flash 4.0 camera (Hamamatsu Photonics). Movie of sequential 1000 frames was acquired by MetaMorph software (Molecular Device) at a frame rate of 30 ms under continuous

illumination. The observation stage was kept at a constant 37 °C, 5% CO2, and humidity (Tokai-hit live-cell chamber and GM-8000 digital gas mixer).

2.2.2. Detection of single fluorescent particle

Subpixel accuracy positions of the PA-GFP dots were determined using the image-processing software u-track (Jaqaman et al., 2008; Rogers et al., 2007) and the accuracy for determining the position of fluorescent dots was estimated using the FIONA method (Ober et al., 2004; Thompson et al., 2002; Yildiz et al., 2003). With this procedure, the trajectory of each fluorescent dot was obtained. We calculated the displacement and the mean square of displacement (MSD) of fluorescent nucleosomes from the tracking data. The originally calculated MSD was in two dimensions. To obtain the 3D value, the two-dimensional value was multiplied by 1.5 ($4D$t / $6D$t). Using KaleidaGraph (Synergy Software, USA), histograms of the displacement were prepared.

Histone H4-PA-GFP has some flexible regions, including the linker and histone tail, which is maximally 50 amino acid residues, corresponding to a length of ~17 nm. The previous study observed by FCS rapid movement of free GFP in the chromosomes at 15 mm^2/s (Hihara et al., 2012). If PA-GFP is rapidly mobile within a restricted area, like a ‘‘dog on a leash,’’ we consider that the effect of the flexible region on the nucleosome position determination is negligible.

2.3. Results and discussion

To observe single nucleosomes and analyze their local dynamics in living human cells, histone H4 was fused with photoactivatable (PA)-green fluorescent protein (GFP) and expressed in DM cells at a very low level (Figure 2.1 A) For single nucleosome imaging, an oblique illumination microscope (Figure 2.1 B) was used to illuminate a limited thin area within the cell (Nikon laser TIRF microscope system Ti with sapphire 488-nm laser). Generally, PA-GFP exhibits green fluorescence only after activation by a 405-nm laser. However, we unexpectedly found that a small fraction of H4-PA-GFP in DM cells was spontaneously activated without laser stimulation, and were observed as dots (Figure 2.1 A and C).

Each bright dot in the nucleus represents a single H4-PA-GFP in a single nucleosome (Figure 2.1 C). We observed single-step photobleaching of the H4-PA-GFP dots (Figure 2.1 D), although photobleaching of multiple H4-PA-GFP complexes should occur in multiple steps or gradually.

The estimated number of H4-PA-GFP was less than 5% of endogenous H4 (Kimura and Cook, 2001). In addition, since free H4-PA-GFP should have a much larger D value, we could trace only the H4-PA-GFP fraction that was stably incorporated into nucleosomes. To show the displacement distribution, we measured approximately 20,000 signal points with single nucleosome tracking with H4-PA-GFP from seven DM cells at a video-rate (~30 ms/frame) as a movie. For single nucleosome tracking, we used u-track software in MATLAB (Jaqaman et al., 2008; Rogers et al., 2007). The dots were fitted to an assumed Gaussian point spread function to determine the precise centers of the dots with higher resolution. We were able to analyze the behaviors of the nucleosomes over short periods of time, ranging from 0 to 0.18 s.

Local nucleosome fluctuation was observed (~50 nm/30 ms), presumably caused by Brownian motion. Three representative trajectories of fluorescently tagged single nucleosomes are shown (Figure 2.1 E; scale bar = 100 nm). Recently, the McNally group also published single-nucleosome tracking data using H2B-EGFP (Mazza et al., 2012), which appear to be consistent with our single nucleosome tracking with H4-PA-GFPs.

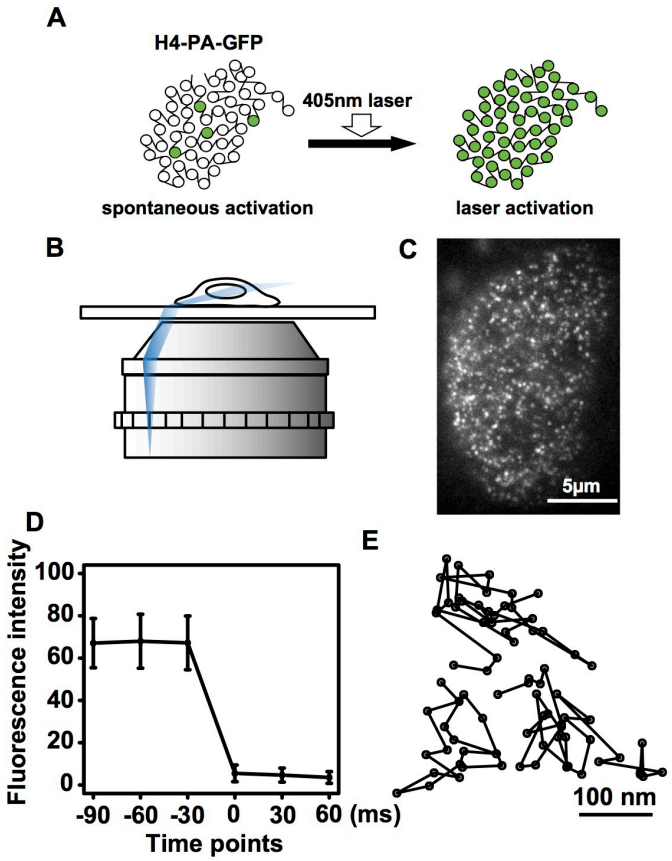

Figure 2.1 Single nucleosome imaging

(A) Generally, PA-GFP shows GFP fluorescence only after activation by a 405-nm laser (right). However, a small fraction of H4-PA-GFP was spontaneously activated without laser activation (left) and was used for our single-nucleosome imaging. (B) A schematic representation of the oblique illumination microscopy (Tokunaga et al., 2008). We used Nikon laser TIRF microscope system Ti with Sapphire 488-nm laser (Coherent). Using a sheet light (blue), a limited area in the cell is illuminated. (C) Single-nucleosome image of a DM cell nucleus expressing H4-PA-GFP observed as a bright dot using the oblique illumination microscopy system. For single-nucleosome tracking, the free software, u-track, was used. The dots were fitted to an assumed Gaussian point spread function to determine the precise center of signals with higher resolution. (D) Single-step photobleaching of the H4-PA-GFP dots. (E) Representative trajectories of fluorescently tagged single nucleosome.

The displacement data of purified GFP fixed on a glass surface were obtained (Figure 3.2 C and D). The average displacement values were 12.8 ± 0.2 nm/30 ms, which was much smaller than those of H4-PA-GFP observed in living cells (59.0 ± 0.2 nm/30 ms in Figure 2.2 A). To evaluate the contribution of whole cell or nuclear movements to the single nucleosome displacements, we again calculated the centroid movements for many nucleosomes in the same time flame (illustrated in Figure 2.3 A). The centroid movements (5.5 ± 0.1 nm/30 ms) (Figure 2.3 B) are much smaller than those in Figure 2.2 A and 2.2 B, suggesting that the detected nucleosome movement is not derived from the global motion of cells or nuclei.

To analyze local nucleosome movement in DM cells further, the MSD values (μm^2) of nucleosomes in the DM cells and fixed EGFP were plotted with their standard errors (Figure 2.3 C). The plots for the nucleosomes fitted well with the exponential equation, $MSD = 0.022t^{0.36}$ (Figure 2.3 C). These results support the restricted nucleosome movement model.

It was reported that movements of large chromatin domains tagged with LacO-array/LacI-GFP (~100 kb and above) were on the 10 and 100 nm length scale for 30 ms (e.g., (Levi et al., 2005)). We could not exclude the possibility that movements of larger domains might contribute to the local nucleosome fluctuation that we observed. Their relationship would be an intriguing issue for further study. This study revealed local nucleosome fluctuation in living mammalian cells (Figure 2.4). Monte Carlo computer simulations suggested that nucleosome fluctuation facilitates the mobility of diffusing proteins in the environment (Hihara et al., 2012). In addition, such nucleosome fluctuation can expose DNA sequences to the surface of chromatin domains more often, while static regular folding structures, such as 30-nm chromatin fibers, can hide or mask most DNA sequences (Figure 2.4). It should be emphasized that both facilitation of protein mobility and DNA exposure lead to an increase in chromatin accessibility. This is in good agreement with our finding that tight cross-linking of nucleosomes blocked antibody accessibility and targeting in dense chromatin regions (Hihara et al., 2012).

Recently, we suggested that interphase chromatin forms numerous compact chromatin domains resembling “chromatin liquid drops” in interphase cells (Joti et al., 2012; Maeshima et al., 2010). This chromatin liquid drops view is in line with predictions of the chromosome territory-interchromatin compartment (CT-IC) model (Cremer and Cremer, 2001; Cremer et al., 2000). In the CT-IC model, each CT is built up from a series of interconnected, megabase-sized chromatin domains, which were originally identified using a pulse labeling as the DNA replication foci (Albiez et al., 2006; Berezney et al., 2005; Schermelleh et al., 2001). Recent high-throughput 3C studies, such as Hi-C and 5C, have also proposed physical packaging of genomic DNA, termed “topologically associating domains” (Nora et al., 2012), “topological domains” (Dixon et al., 2012) or “physical domains” (Sexton et al., 2012). Local nucleosome fluctuation would play an especially important role in such compact chromatin domains because the effect of nucleosome fluctuation on the facilitation of chromatin accessibility is more significant in a compact chromatin environment (Hihara et al., 2012).

Many biological processes, including transcription, DNA repair, replication, and recombination, are based on “scanning genomic DNA and targeting” (Figure 2.4). For example, in transcriptional regulation, the dynamic movement of nucleosome fibers assists with the targeting of transcription factors and complexes. Dynamic irregular folding can easily form loops, facilitating interaction between promoters and enhancer sequences. Since the flexible and dynamic nature of nucleosome fibers in living mammalian cells drives such biological processes, regulation of local nucleosome dynamics, possibly by histone modification and/or specific proteins, will be an important factor in their regulation.

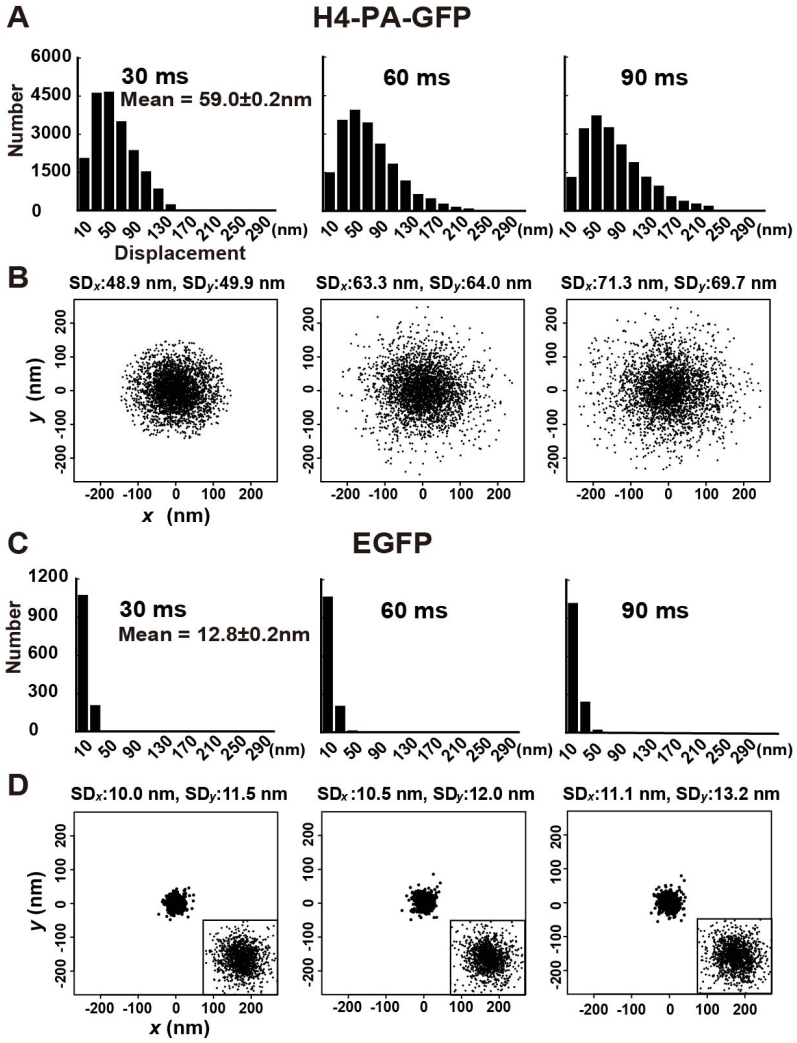

Figure 2.2 Single nucleosome analysis of H4-PA-GFP

(A) Displacement (movement) histograms of single nucleosomes in interphase DM cells for 30 ms (left), 60 ms (center), and 90 ms (right; n = 20,000 in seven cells). The mean displacement ± standard error for the 30 ms time point was indicated. (B) Distribution of nucleosome displacements in the *x*-*y* plane for 30 ms (left), 60ms (center), and 90 ms (right). n = 3,000 in seven cells. Standard deviations (SD_x and SD_y) were shown. In panels (A) and (B), the originally calculated displacement were in two dimensions. To obtain their 3D values, the two-dimensional values of displacement were multiplied by $\sqrt{1.5}$ ($\sqrt{(6Dt/4Dt)}$). (C) Displacement histograms of single EGFP on a glass surface for 30 ms (left), 60 ms (center) and 90 ms (right; n = 1,300). The mean ± standard error for the 30 ms time point was indicated. (D) Distribution of EGFP displacements in the *x*-*y* plane for 30 ms (left), 60ms (center), and 90 ms (right; n = 1,300). Standard deviations (SD_x and SD_y) were shown. The insets are 3-fold magnified images of the distributions.

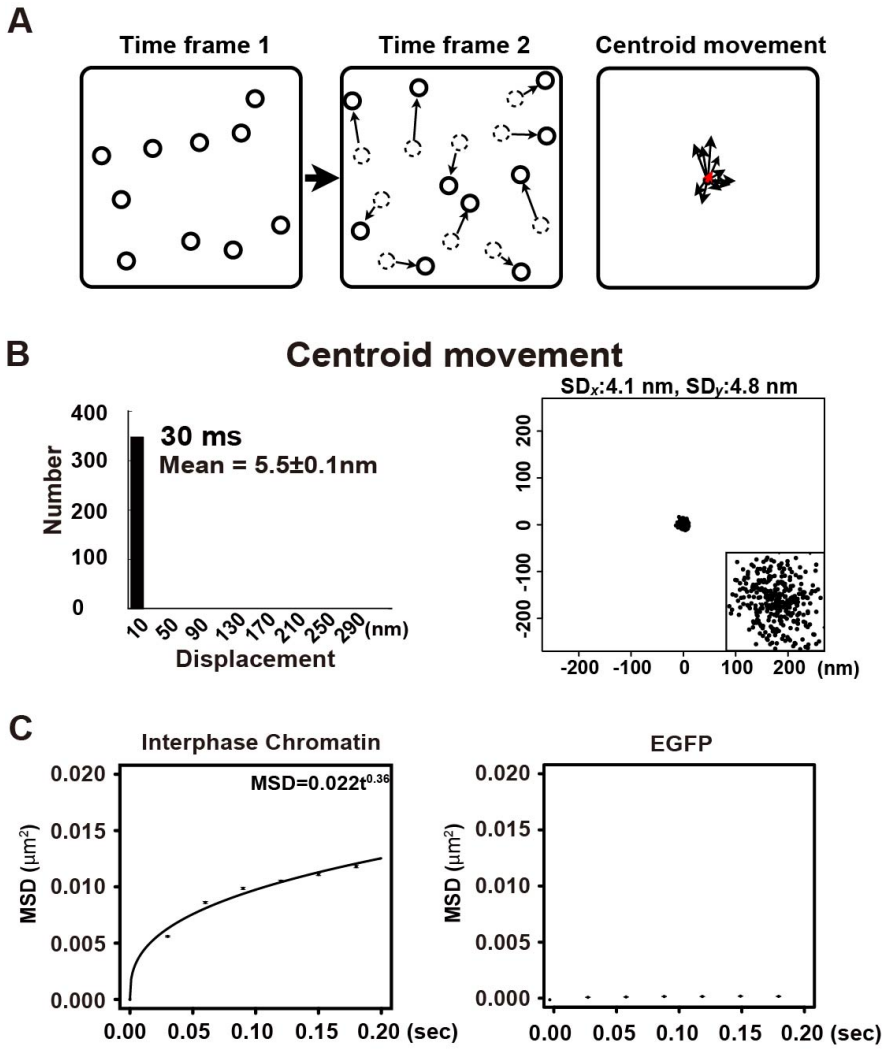

Figure 2.3 Local nucleosome fluctuation in living mammalian cells

(A) Schematic representation for calculating centroid movement for many nucleosomes in the same time frame. The black arrows represent the movements of nucleosomes and the small red arrow represents centroid movement in the frame. (B) Displacement histogram (left) and distribution in the *x*-*y* plane (right) of centroid movement in the frame for 30 ms (n = 350 in seven cells). The inset is 10-fold magnified image of the distribution. (C) Plots of the mean-square displacements (MSDs) with standard errors of single nucleosomes in interphase chromatin (left) and single EGFPs on a glass surface (right) from 0 to 0.18 s. The plots for single nucleosomes were fitted as an anomalous diffusion, suggesting that nucleosome movement supports a restricted diffusion model. Error bars represent the standard errors of the mean value. In the panel of MSD for single nucleosomes, the originally calculated MSD were in two dimensions. To obtain their 3D values, the two-dimensional values of MSD were multiplied by 1.5 ($6Dt$ / $4Dt$).

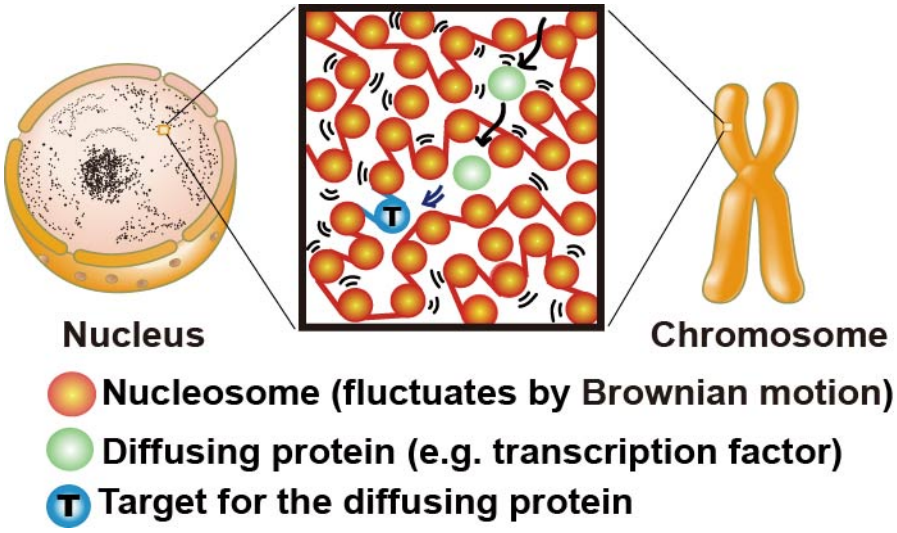

Figure 2.4 Flexible and dynamic nucleosome fiber in living mammalian cells
We demonstrated the flexible and dynamic nature of the nucleosome fiber in living mammalian cells with the newly developed single nucleosome imaging. Since the flexible and dynamic nature of nucleosome fibers in living mammalian cells drives biological processes, regulation of local nucleosome dynamics, possibly by histone modification and/or specific proteins, will be an important factor in their regulation.

3. Chapter 4: Dynamic chromatin domains are organized by coordination of cohesin and nucleosome-nucleosome interactions in living mammalian cells

3. Summary of results and discussion

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4. Chapter5: Conclusions

4.1. Summary

In this thesis, I mainly discussed chromatin and epigenetics. First, we analyzed epigenetic regulation and promoter types based on genome-wide ChIP-Seq and DeepCAGE data. Secondly, we newly developed the single nucleosome imaging method that can be implemented in a living nucleus. Lastly, we succeeded in observing chromatin structure and dynamics at single nucleosome level and their variation related to different types of histone modifications and chromatin associated proteins.

Currently, to tackle the epigenetic phenomena, we can utilize the tool “ChIP-Seq” that detects the histone modification patterns genome-widely. We performed a systematic analysis of transcription promoters and gene expression, as well as of epigenetic histone behaviors containing several types of histone modification and genomic positioning. We found that, in humans, broad promoters, which initiate transcription from a wide-ranging region, but not peak promoters which initiate transcription from a narrow genomic region, generally had significant associations with histone modification and nucleosome position. Specifically, around broad promoters histones were highly distributed and aligned in an orderly fashion. This feature was more evident with histones that were methylated or acetylated; moreover, the nucleosome positions around the broad promoters were more stable than those around the peak ones. Nucleosome positioning and transcriptional factor binding results support the strong connection between the nucleosome-free region and the accessibility of transcription factors, which was specific to broad promoters. More strikingly, the overall expression levels of genes associated

with broad promoters (but not peak promoters) with modified histones were significantly higher than the levels of genes associated with broad promoters with unmodified histones. These results shed light on how epigenetic regulatory networks of histone modifications are associated with promoter architecture (Chapter 2).

ChIP-Seq analysis is adequate to the correlation analysis of epigenetic regulation and gene expression, but is insufficient to investigate chromatin environment and the process of epigenetic regulation, therefore, the tool to directly observe the chromatin structure and dynamics is required. Then, we achieved to develop the single nucleosome imaging method and visualize chromatin structure and dynamics at single nucleosome level. Two tips; simultaneous activation of PA-GFP or PA-mCherry without 405 nm laser activation and the oblique illumination enabled us to observe the single molecules in the nucleus. The mean square displacement of nucleosomes showed abnormal diffusion and suggested that movement of nucleosome restricted by DNA. Further, we carefully evaluated the validity of single nucleosome imaging methods compared to EGFP or the movement of the nucleus. These results show the flexible and dynamic structure of chromatin and chromatin is fluctuating in the nucleus (Chapter 3).

Although previous studies had been proposed static chromatin nature, our result suggested dynamic nature of chromatin. Newly developed imaging method of single nucleosomes overall a living nucleus enabled us to reconstruct the super-resolution image of chromatin. Super-resolution image of chromatin showed clustered pattern of nucleosomes and statistic analysis showed the existence of chromatin domain but not a random distribution. This result supports the irregular folding model and the average size of radius ~115 nm (i.e. ~230 nm diameter) consistent with the previous X-ray scattering analysis (Joti et al., 2012). This calculated domain size is similar to that of the domains recently identified by high-resolution Hi-C method (~180 kb, ~248 nm diameter) (Rao et al., 2014) if we assume that the nucleosomes occupy ~30 % of the domain space, suggesting that both methods observed similar domain structures. Since TADs or chromatin domains are functional units of the genome, keeping these units throughout cell cycle will be efficient for the cells. This point is also the case for the memory of epigenetic marks on the chromatin domains during cell cycle. Subsequently, our results propose a novel mechanistic insight into mitotic chromosome assembly: the chromatin domains formed in the interphase are assembled together to create a rod-like chromosomal shape during mitosis and chromatin domains function as building blocks of chromosomes. Furthermore, we succeeded in creating “heat-map”, revealing chromatin movement on the local position of nucleus and the more heterochromatic regions, the less chromatin movement. Heatmap and MSD analysis shows that chromatin domain was fluctuated by alteration of histone modification and hypotonic condition and depletion of chromatin associating proteins. Especially, TSA that inhibit the histone deacetylation and increase acetylated marks shows the decondensation of chromatin domain and increase of chromatin mobility. Additionally, heterochromatin region enriched with methylated histones shows the condensed pattern and low mobility of chromatin, and through the differentiation of ES cells, chromatin becomes condense and decrease the mobility. These results suggested that the change of chromatin environment at single molecule level is related to histone modification patterns (Chapter 4).

4.2. Future directions

Now we have collected the experimental techniques ChIP-Seq, Hi-C, and single nucleosome imaging required for chromatin and epigenetics study. As the future direction, let us see the list of remaining problems.

1. The direct function of chromatin environment
2. The relationships between chromatin environment and various histone modification types
3. Chromatin environment on specific genomic loci
4. Histone modification imaging in a living cell
5. Genotype, epigenotype, and phenotype

In this thesis, we revealed the variation of chromatin structure and dynamics at euchromatic region and heterochromatic region, suggesting the difference of chromatin environment is directly related to the cellular function. To answer "1. The direct function of chromatin environment", combined single nucleosome imaging with single molecule imaging of proteins that interrogate the target site, we will investigate how the chromatin environment interacts with the protein and has a function.

Then, we observed methylated or acetylated histones contributed to the chromatin environment, but currently numerous histone modification types are assumed to be interact with the chromatin environment (Strahl and Allis, 2000). To answer to "2. The relationships between chromatin environment and various histone modification types", based on the knowledge of histone modifier or binding proteins on modified histones, knock-out or knock-down experiments of such proteins will become powerful.

We observed chromatin environment in the total nucleus, therefore, to further understand the detail of chromatin we should observe chromatin structure and dynamics on specific genomic loci, especially around the target gene. To answer "3. Chromatin environment on specific genomic loci", we can mention TALEN and CRISPR system (Chen et al., 2013; Miyanari et al., 2013), but at present it is not sufficient and needs to be improved the background level.

Furthermore, it is hard to observe the just only specifically modified histones in a living cell. To answer "4. Histone modification imaging in a living cell", we have to improve the method to observe the specific histone modification based on Fab or the other technique (Stasevich et al., 2014).

The biggest question is to reveal the relationships among "genotype, epigenotype, and phenotype". Epigenotype is the bridge between genotype and phenotype, so the necessity of chromatin and epigenetics research arises.

4.3. Conclusion

Chromatin led the 20th century's biological research, and epigenetics is now one of the most popular areas in the 21th century's biological research and the largest price of post-genomic era. Although the majority of epigenetic research is related to histone modification and DNA methylation, the research that makes the bridge between chromatin and epigenetics is not sufficient. Epigenetics is one of the most important concepts to understand the highly complicated eukaryotic body but the types of histone modifications and related regulations are diverse and complicated. Although this appears the diverse aspects of living organisms, we need to describe more generalized phenomena for understanding. In order to do that, understanding of chromatin that is the vessel of epigenetic components is essential. I believe that our results by imaging and bioinformatics approach will provide a basis of dynamic and flexible nature of chromatin toward understanding the function of chromatin in eukaryotic epigenetic system and stimulate the mind of researchers.

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