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Topokaryotyping – a proposal for a novel approach to study nuclear organization

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Gene positioning in the nucleus plays an important role in gene activity and genome stability, both in norm and pathology. We propose a new principle for the analysis of the large scale chromatin organization at the single-cell level, termed Topokaryotyping. It is based on coexpression in cells of (i) biotin ligase BirA targeted to a particular intranuclear domain (via its fusion with a protein-marker of this domain) together with (ii) BAP-histone fusion. The application of biotin pulse chase followed by (i) generation of mitotic spreads, (ii) detection of the biotin label and (iii) karyotyping technique to identify the chromosomes should provide information on the association of particular chromosome regions with the nuclear domain under study. We discuss potential advantages of the proposed approach as compared to other methods to study genome topology in the nucleus.

Keywords: chromatin, single-cell analysis, biotinylation, nucleus, genome topology, epigenetics

Organization of genome in the nucleus and the methods of its study

Recent progress in the postgenomic era demonstrates that the complete knowledge of genome sequence is not sufficient to fully understand its function in cells. Cells (and nuclei) are not biochemical reactors, governed by the equations of chemical kinetics of reactions in homogenous solutions. Rather, they are more machine- or clock-like systems, i.e., highly structured and organized in space, with the mutual positions of parts, their orientations and interactions playing a crucial rule.

In regards to the eukaryotic genome itself, it is packed into chromatin – a hierarchical structure composed of DNA, histones and various non-histone proteins. Several levels of chromatin compaction are known, and the information about every level of this organization – from the nucleosome positioning to its higher level folding and ultimately to the location of a particular genomic sequence in the nucleus (topological organization of genome) – appears to be important for the understanding of various aspects of a gene function, including transcription, replication, repair and recombination [1].

In higher eukaryotes, the topological organization of the genome within the nucleus was shown to be nonrandom [2, 3], with chromosomes and individual genes occupying preferential positions relative to: (i) each other, (ii) various nuclear compartments and (iii) nuclear periphery [3, 4, 5].

Many evidences suggest that gene positioning in the nucleus plays an important role in gene activity and genome stability [6]. The active genes are often located in the center and silent genes at the periphery of the nucleus [7]. Changes in gene location occur during physiological processes such as differentiation and development [5, 8] and can also lead to different pathological states [9, 10, 11]. In particular, the study of gene locations in the nucleus appears to be important in oncology, at least for two different reasons: i) in various cancer types including breast and pancreas, the position of genes relevant for cancerogenesis was shown to change and correlate with

their transcriptional activity [12, 13, 14] and ii) the intra-nuclear proximity of different chromosomes to each other affects the frequencies of chromosome translocations responsible for different cancer types such as Chronic myelogenic leukemia [6, 15].

Although it is difficult to study the highest (nuclear) level of genome organization in cell free systems, numerous ways to analyze it in the context of living cells have been developed. One can distinguish between (i) biochemical and (ii) single-cell level studies of chromatin organization in the nucleus.

(i) Biochemical approaches either require a protein associated with a particular nuclear domain, such as chromatin immunoprecipitation (ChIP) [16, 17] or DamID [18], or can work solely at the genomic level, such as various versions of chromosome conformation capture: 3C, 4C, 5C, HiC etc [19, 20, 21, 22]. ChIP with the antibodies targeted to a particular nuclear domain with consequent analysis of associated DNA allows one to infer the proximity of a given sequence to this domain. An alternative approach, called DamID [18] employs fusion of the protein of interest to DNA methyl-transferase and analysis of methylated DNA, and was used successfully to identify nuclear envelope associated sequences. Various CC versions are all based on the analysis of spatial proximity between different DNA sequences in the nucleus after their ligation in the context of nuclear structure [19, 20, 21, 22].

An advantage of these approaches is their relatively high resolution, which could reach nucleosome scale for chromatin immunoprecipitation (i.e., 150 bp) and 1kb for the latest versions of chromosome conformation capture [23]. However, the main shortcoming of the biochemical approaches is the need to work with many cells, which yields information averaged over a large cell population. The averaging might lead to the loss of information about correlations between the states of different parts of genome in individual cells.

Most of the cell populations are heterogenous and thus require single-cell level analysis for comprehensive understanding. A telling illustration that there is a real need in such analysis is one of the biggest challenges in oncology of today – the tumor heterogeneity – with the role of a small subfraction of cancer cells, called cancer stem cells, receiving an increasing recognition [24]. Given that the cell variations in tumor could have both genetic as well as epigenetic nature, the understanding of the variability of genome organization at the nuclear level in individual cells should be beneficial for both fundamental and clinical research. Single cell level analysis is typically performed with microscopy and is based of in situ hybridization (FISH), which allows one to detect the position of a particular gene in the nucleus [25]. A variation of FISH, termed chromosome painting allows one to locate whole chromosomes in the nucleus [26, 27], and its more advanced versions SKY (spectral karyotyping) [28] and M-FISH (multiple color FISH) [29] are able to locate each chromosome in the same nucleus. More recently, different proteins designed to recognize a specific genomic region (zinc finger [30, 31], TALEN [32] and Cas9-based [33]), fused with a fluorescent proteins became also available, allowing one to monitor the intranuclear location of the sequence of interest and its changes in living cells.

Here, we will describe a proposal to develop a principally new methodology to analyze the intranuclear position of genome loci at the single cell level, which is complementary to the existing FISH-based methods, as well as fluorescent protein fusions with sequence specific-targeting domains. This approach, which we propose to term Topokaryotyping represents a logical continuation of the *in vivo* protein biotinylation technology that our group worked on over the years. The next section will briefly describe the main steps in the development of the latter technology.

In vivo biotinylation and Proximity Utilizing Biotinylation

The principle of in vivo biotinylation

In vivo biotinylation was first developed as an alternative to an epitope tagging, a widely used method to circumvent an often cumbersome and resource-heavy procedure of developing special antibodies for the protein under study [34] and to make its detection and purification possible with standard re-

agents. In this regard, the biotin/avidin system has a special interest for affinity-based purification and detection methodologies. The interaction between biotin and streptavidin is the strongest known non-covalent interaction, with a dissociation constant of 10^{-15} M, which is several orders of magnitude higher than that of commonly used affinity tags. Biochemistry-wise, it was interesting to take advantage of this strong interaction, which allowed one to use stringent washing conditions leading to reduction of the experimental background. In addition, there are very few naturally biotinylated proteins, which reduces the chance for cross-reaction, as opposed to antibodies which usually cross-react with several species of proteins. Finally, many commercially available reagents for purification and detection of biotinylated macromolecules have been developed over the years. Our group has set up a system for the expression of biotinylated proteins of choice in mammalian cells *in vivo*. It is based on the coexpression of the protein of interest fused to a short biotin acceptor peptide (BAP), together with the biotin ligase BirA from *E. coli* that targets very specifically this peptide. We showed that this system ensures an efficient biotinylation, and that the use of biotin as an epitope *in vivo* does not generally disturb the intracellular localization of the tagged protein. Similar technique was also published by the group of Strouboulis [35] and later was used in such applications as Western detection, study of protein-protein interactions and immunofluorescent microscopy.

In addition, our group demonstrated that *in vivo* biotinylation can have more specialized applications.

Chromatin immunoprecipitation (ChIP)

One of the biggest problems with ChIP analysis is a 'sticky' nature of chromatin, leading to high level of non-specific binding and the need to develop ways to reduce it. We showed that *in vivo* biotinylation allows the use of more stringent washing conditions, compared to those employed in a regular ChIP protocol. This feature significantly improves the signal to noise ratio, as judged by quantitative PCR analysis [34]. As a result, our system helps to streamline the ChIP procedure and make it more robust and economical.

Immunoelectron microscopy

The same technique was also shown to have a number of advantages for immunoelectron microscopy. The immunostaining in electron microscopy experiments is typically compatible only with formaldehyde cross-linking, which does not preserve cell morphology well. We have demonstrated that detection of biotin (by streptavidin or anti-biotin antibodies) is compatible with a wider range of post-embedding methods, facilitating combination of morphological and localization studies in a single experiment. We also showed that the method works in both transient transfection and stable cell line expression protocols, and thus can be used for colocalization studies [36].

Humanized BirA

In a parallel development, a codon-optimized version of BirA ligase was also developed in our group, which improved its expression in mammalian cells and thus the efficiency of biotinylation of the target proteins of interest [37]. This particular reagent became highly requested. Over the years, the group has received over 200 requests from different laboratories around the world for this construct. In particular, the BioID method [38] was developed using the humanized BirA provided by our group.

Proximity Utilizing Biotinylation (PUB)

In the course of our experiments, we have sometimes observed that the biotinylation efficiency of a particular BAP-protein fusion was compromised by its low accessibility for BirA targeting. This could happen, for example, when the BAP-protein was localized in a specific intracellular compartment. Based on this observation, our group has undertaken to revamp the *in vivo* biotinylation technique in order to develop an alternative approach to study protein-protein interactions and proximity *in vivo*, named Proximity Utilizing Biotinylation (PUB). It is based on coexpression of protein X fused to biotin ligase (BirA) and protein Y fused to biotin acceptor peptide (BAP). If the proteins are in proximity or interact with each other, BirA will specifically biotinylate BAP do-

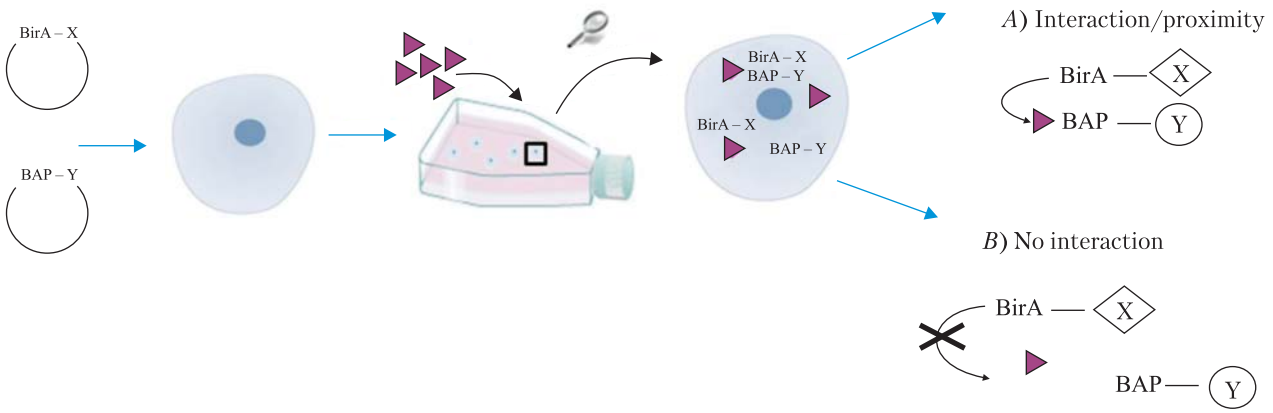

Fig. 1. PUB Principle. Proximity Utilizing Biotinylation approach is based on coexpression of protein X fused to biotin ligase (BirA) and protein Y fused to biotin acceptor peptide (BAP). *A.* -When the proteins are in proximity to (or interact with) each other, BirA will specifically biotinylate BAP domain. Result of an efficient biotinylation of BAP domain is a biotin signal, which can be detected by different methods, such as Western blot, immunofluorescence and mass spectrometry. *B.* - If two proteins are not in close proximity or they do not interact, one should expect a significantly lower biotinylation background, due to protein mobility in the cell and their random collisions

main (Figure 1). Similar approach was also suggested by Ting group [39], however we proposed different applications of this technique, described below.

PUB-MS: The common techniques for the study of protein-protein interactions *in vivo* are not well adapted to the capabilities and the expertise of a standard proteomics laboratory, typically based on the use of mass-spectrometry. The PUB-MS method was developed with the aim to close this gap. In particular, we have redesigned the sequence of BAP to make it more mass-spectrometry friendly (i.e., to produce a peptide with good ionization properties after trypsin digestion). We confirmed that the biotinylation *in vivo* is specifically enhanced when the BAP- and BirA- fused proteins interact or are in proximity with each other. The advantage of the mass-spectrometry detection methods of BAP biotinylation was demonstrated by using BAPs with different sequence in one experiment (allowing for multiplex analysis) and by the use of stable isotopes [40].

PUB-NChIP: In the course of this work, we have also realized that our technique can be used to label a specific subfraction of a BAP-protein of interest that is close to given BirA-fusion, and to study its localization or biochemical properties. As one application of this idea, we have developed PUB-NChIP (Proximity Utilizing Biotinylation with Native ChIP [41]) to purify and analyze protein composition of chromatin in the proximity to a nuclear protein of interest. It is based on coexpression of a) a nuclear protein of interest, fused with BirA together with b) a histone fused to BAP. We demonstrated that chromatin is specifically biotinylated in the proximity of the BirA-fusion and can be further purified biochemically. Unlike a usual ChIP method, no chemical cross-linking is involved in the process (which usually damages the histone part of chromatin), which allows one to study the post-translational modifications with the PUB-NChIP method. This method also has additional advantages. First, one can use BAP-fusions with alternative histones (such as H2AZ, macroH2A, H2ABBD, H3.3, CenpA), instead of the canonical ones. Given that alternative histones have been shown to preferentially associate with different functional states of chromatin, PUB-NChIP should provide a more fine-grained analysis of chromatin organization in living cells, as compared to regular ChIP.

Topokaryotyping

The distinctive feature of proximity biotinylation is that the BAP-protein in proximity to the BirA fusion is left with a permanent molecular mark (i.e. biotin), which can persist after the proximity between BAP-

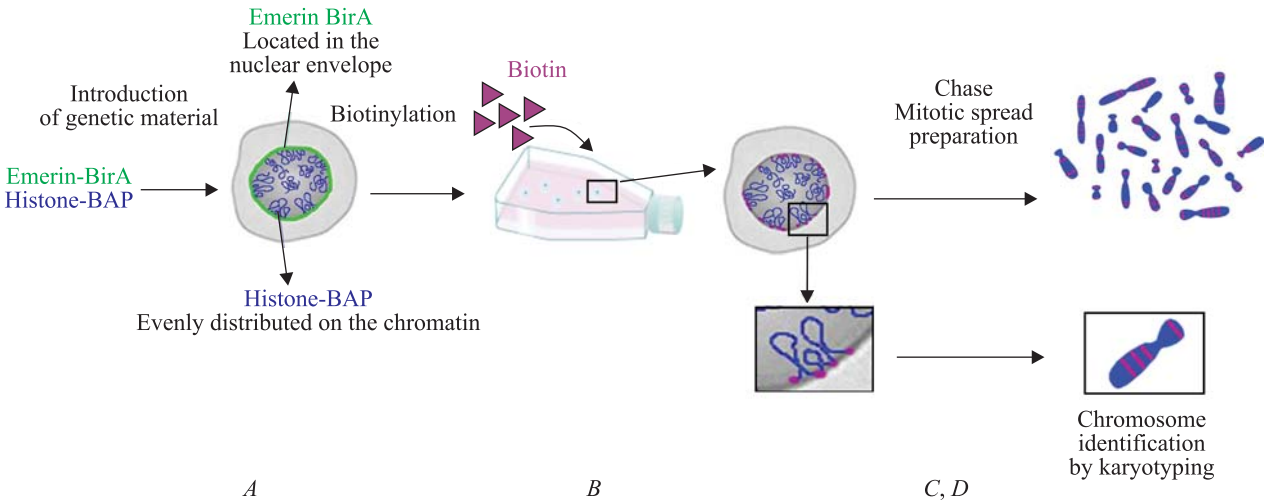

Fig. 2. Topokaryotyping principle, illustrated by labeling of chromatin due to its proximity to nuclear envelope (NE). *A*. BirA-fusions of NE-associated proteins (such as emerin or laminB) are expressed together with BAP-histone fusion. BirA is thus targeted to the nuclear envelope, while histone-BAP is evenly distributed in the nucleus. *B*. After pulse-biotinylation, the parts of the chromatin that were close to the nuclear envelope in the interphase acquire a permanent biotin mark on the histone-BAP due to their proximity with the Emerin-BirA. *C*. After washing the cells of biotin and allowing them to enter mitosis, the metaphase chromosome spreads are obtained and the biotinylation is revealed (e.g., with streptavidin cy3 (red)), while the total DNA could be counterstained with DAPI (blue) or another dye of choice). Given the limited number of chromosome domains proximal to the NE, we expect that this staining will not be homogenous and instead will be manifested as discrete bands. *D*. The identification of the chromosomes and band positions with a karyotyping technique will allow one to infer which parts of genome in this particular cell were close to the nuclear envelope in the interphase

and BirA-fusions has been lost. Given that most of the proteins have an average half life of 1-2 days and some are stable for much longer, one can take advantage of this feature and, by performing pulse-biotinylation followed by chase, be able to monitor the state of a protein of interest (fused with BAP) at a defined time after its interaction with another protein has occurred. In particular, we have shown that one can label chromatin in proximity to repair-relation protein Rad18, localized in characteristic foci in cell nuclei. Whereas Rad18 signal and biotinylation signal perfectly colocalized after pulse-labeling with biotin, the colocalization was progressively lost after the biotin removal and several hours of chase [41].

This observation logically leads us to the new methodology, also based on the Proximity Utilizing Biotinylation, which we propose to develop. Similar to PUB-NChIP, this methodology will use BAP-histone fusions for labeling of chromatin in specific locations of the nucleus. However, instead of biochemical purification of biotinylated chromatin, we propose to use microscopic detection of biotinylated regions on mitotic chromosomes. In what follows, we describe the main principle and potential applications and advantages of this approach.

The main idea is to use the fact that the biotin label persists on the chromatin in living cells for a relatively long time. Importantly, our observations indicate that this time is sufficient to allow the cell to enter mitosis and for the interphase chromatin to form mitotic chromosomes, still retaining the biotinylation mark, introduced in particular chromatin regions due to their proximity to the BirA-fusion of choice.

Accordingly, the combination of the following steps (Figure 2): (i) PUB-labeling chromatin by a BirA-fusion of a protein that is targeted to a particular nuclear domain in interphase, (ii) driving cells into mitosis and (iii) staining the mitotic chromosomes for biotin, – is expected to reveal the chromosome regions corresponding to the regions of genome that were in proximity to a particular location in the interphase nucleus. The next (iv) step is the combining of this

method with a karyotyping technique, which would allow one to identify the chromosomes and thus point to the genomic identity of these regions. As a result, one should be able to infer the intranuclear location of particular regions of genome in the interphase nucleus. Importantly, as individual mitotic spreads are analyzed, this approach would allow one to perform this analysis at the single cell level.

We propose to name this new approach Topokaryotyping, as it is expected to allow us to use karyotyping technique to analyze genome topology at the single cell level. Next, we briefly describe the advantages of this novel technology, as compared to the existing methods to study nuclear organization.

(i) Chromatin Immunoprecipitation – As the proposed method is based on the analysis of individual chromosome spreads, it should permit one to analyze genome topology at the single cell level, unlike the biochemistry-based ChIP approach. Although the resolution of this method might be lower than what is achieved by ChIP, extended chromatin fiber technique [42] and detection of biotinylated histones, combined with FISH (e.g., chromosome painting) could lead to improvements in this regard.

(ii) CC approaches – the single cell level of analysis is an advantage of the Topokaryotyping approach, but the resolution would be typically lower. On the other hand, one might conceive of an application of Topokaryotyping that would be complementary to the 3C technique. By targeting BirA to a particular genome region (via zinc finger, TALEN or Cas9-based methods), one can label chromatin in physical proximity to this genome region and identify it. This may be useful for identification of potential translocation partners in cancer (see e.g., [15]).

(ii) Regular FISH and fluorescent protein fusions – Topokaryotyping should provide a more global view on the genome localization, without the need to select specific gene regions for the analysis.

(iv) SKY and multiFISH – As particular bands of chromosomes will be labeled, Topokaryotyping should provide a better resolution than these chromosome-painting-based methods.

Finally, we offer several clarifications concerning this technique.

In regards to the implementation of this approach, it should be possible to introduce the BirA- and BAP- fusions in the cells by a number of methods, including transient transfection of DNA, generation of stable cell lines, RNA transfection or even protein transduction.

Importantly, this approach can be also extended to other than biotinylation methods of protein proximity labeling, such as lipoic acid ligase (LplA) system, engineered by the Ting group [43] provided that the labeling of chromatin is sufficiently stable. Moreover, the use of different colors for label detection will allow one to observe at once the genome regions in proximity to different nuclear domains of choice, making it possible to analyze several nuclear compartments at the single cell level.

We expect that the proposed methodology, despite its relatively low resolution, will extend the toolbox of the available methods to analyze nuclear organization at the single cell level and will find many applications in cellular biology, epigenetic studies and chromatin science.

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Топокариотипирование – принцип нового подхода к изучению ядерной организации

Позиция генов в ядре критична для их функционирования и стабильности генома, в нормальных и патологических состояниях клетки. Мы предлагаем новый принцип анализа организации хроматина на ядерном уровне в индивидуальных клетках, называемый Топокариотипирование. Он основан на совместной экспрессии в клетках (i) Биотин лигазы BirA локализованной в конкретном ядерном домене (за счет ее фузирования (слияния) с белком-маркером этого домена) и (ii) гистонового гена слитого с BAP доменом (пептидом-акцептором биотина). Мечение клеток биотином и их последующая отмывка, за которой следуют (i) создание препаратов метафазных хромосом, (ii) детектирование биотиновой метки на хромосомах и (iii) кариотипирование с целью идентификации хромосом – должны предоставить информацию об ассоциации данного хромосомного региона с исследуемым ядерным доменом. Мы обсуждаем потенциальные достоинства предлагаемого метода с другими подходами к изучению топологии генома в клеточном ядре.

Ключевые слова: хроматин, анализ отдельных клеток, биотинилирование, ядро, топология генома, эпигенетика

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Топокаріотипування – принцип нового підходу для дослідження ядерної організації

Позиція генів в ядрі критична для їх функціонування та стабільності геному, в нормальних і патологічних станах клітини. Ми пропонуємо новий принцип аналізу організації хроматину на ядерному рівні в індивідуальних клітках, званий Топокаріотіпіроване. Він заснований на спільній експресії в клітинах (i) Біотин лігази BirA локалізованої в конкретному ядерному домені (за рахунок її фузірованія (злиття) з білком-маркером цього домену) і (ii) гістонові гена злитого з BAP доменом (пептидом-акцептором біотину). Мічення клітин біотином і їх подальша відмивання, за якою слідують (i) створення препаратів метафазних хромосом, (ii) детектування біотіновой мітки на хромосомах і (iii) каріотипування з метою ідентифікації хромосом – повинні надати інформацію про асоціацію даного хромосомного регіону з досліджуваним ядерним доменом. Ми обговорюємо потенційні переваги запропонованого методу з іншими підходами до вивчення топології генома в клітинному ядрі.

Ключові слова: хроматин, аналіз окремих клітин, біотінілювання, ядро, топологія геному, епігенетика

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