Please cite this article in press as: Agbleke et al., Advances in Chromatin and Chromosome Research: Perspectives from Multiple Fields, Molecular Cell (2020), https://doi.org/10.1016/j.molcel.2020.07.003

Molecular Cell

CellPress

Technology Review

Advances in Chromatin and Chromosome Research: Perspectives from Multiple Fields

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https://doi.org/10.1016/j.molcel.2020.07.003

Nucleosomes package genomic DNA into chromatin. By regulating DNA access for transcription, replication, DNA repair, and epigenetic modification, chromatin forms the nexus of most nuclear processes. In addition, dynamic organization of chromatin underlies both regulation of gene expression and evolution of chromosomes into individualized sister objects, which can segregate cleanly to different daughter cells at anaphase. This collaborative review shines a spotlight on technologies that will be crucial to interrogate key questions in chromatin and chromosome biology including state-of-the-art microscopy techniques, tools to physically manipulate chromatin, single-cell methods to measure chromatin accessibility, computational imaging with neural networks and analytical tools to interpret chromatin structure and dynamics. In addition, this review provides perspectives on how these tools can be applied to specific research fields such as genome stability and developmental biology and to test concepts such as phase separation of chromatin.

Introduction

DNA within eukaryotic nuclei exists as a protein-DNA complex called chromatin. The fundamental unit of chromatin is the nucleosome, composed of approximately 146 bp of DNA coiled around a histone protein octamer (Luger et al., 1997). Decades of research showed that chromatin organization is important to regulate several DNA-based transactions including transcription, DNA repair and replication (Bickmore and van Steensel, 2013; Bonev and Cavalli, 2016; Pombo and Dillon, 2015; Seeber et al., 2018; Sexton and Cavalli, 2015). Yet despite tremendous progress in the field, particularly with regards to transcription and DNA repair, how chromatin impacts development, disease, and evolution remains largely unexplored.

The aim of this collaborative review is to highlight key technologies that can be used to address major questions in chromatin biology (Figure 1; Table 1). These include cutting-edge microscopy techniques to image chromatin organization with super resolution, tools to physically manipulate chromatin, computational imaging tools to interpret chromatin structure and dynamics, and powerful single-cell chromatin accessibility techniques. We include viewpoints from early-stage researchers in adjacent fields that complement chromatin research, for instance, how methods used in protein structure prediction may help solve the chromatin structure prediction problem. Importantly, this review gathers perspectives from not only the laboratories that develop these technologies but also from fields that are ready to exploit them, such as organismal and developmental biology or the recently revitalized field of phase separation. We hope this joint effort will drive further collaborations within the chromatin community, as well as draw new interest from outside fields, to advance chromatin biology.

Imaging Chromatin and Nuclear Proteins

Multiplexed FISH Combined with Super-Resolution Imaging to Study Chromatin Structure

The spatial organization of interphase chromatin and its interplay with various biological functions has been one of the most intriguing open questions in cell biology. Imaging methods such as fluorescence *in situ* hybridization (FISH) have been essential to our understanding of the structure and function of chromatin (Speicher and Carter, 2005). Despite having much

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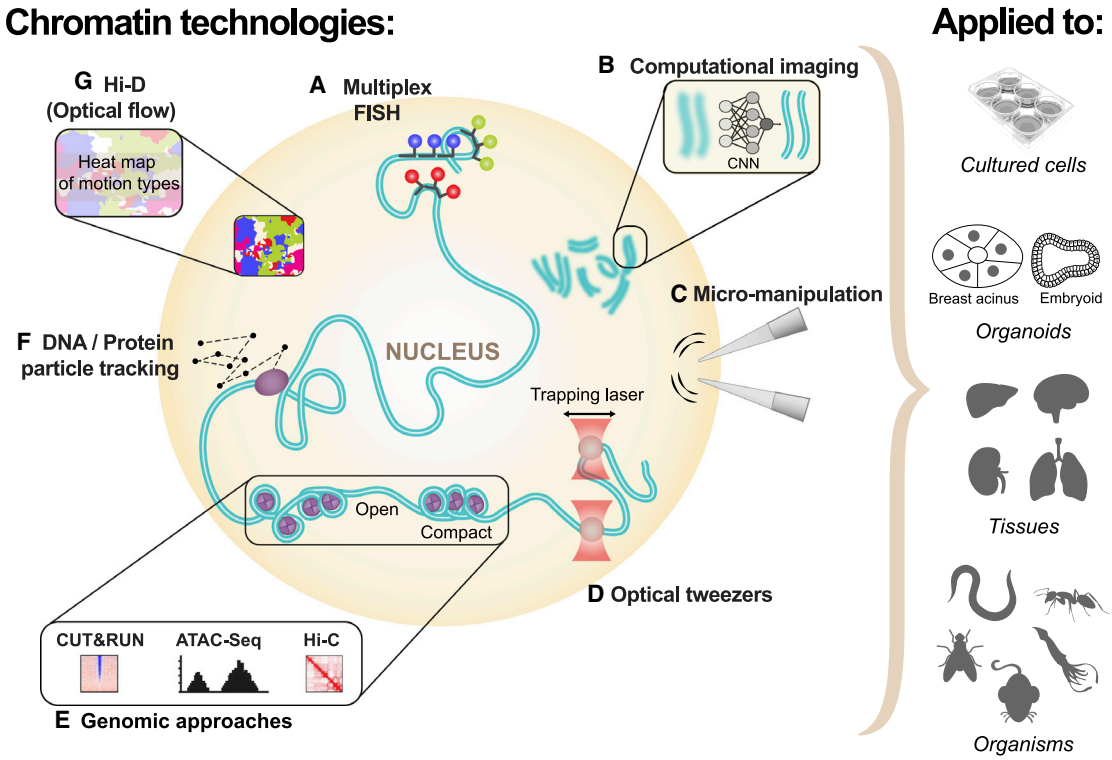

Figure 1. An Overview of Techniques for Exploring Chromatin Discussed in This Review

(A) Multiplexed FISH imaging, when combined with super-resolution imaging, can probe genomic regions corresponding to TADs and, in the future, will be used to trace whole chromosomes.

(B) Using machine learning and neural network architectures, computational imaging, is driving major advances in imaging, e.g., converting a low-resolution image into a high-resolution image using a trained convolutional neural network (CNN).

(C) Micro-manipulation of isolated nuclei using pipettes can be used to measure forces acting on the nucleus and how they change in response to different environmental conditions.

(D) While still in an early phase of development, trapping and manipulation of chromatin using optical tweezers has great potential to be a powerful tool to study chromatin *in vivo*.

(E) A variety of sequencing-based genomic approaches that probe chromatin exist. Arguably the most important of these is Hi-C, which quantifies the number of interactions between genomic loci that are nearby in 3-D space (not discussed in detail in this review). Important complements to this method, are techniques that measure chromatin-protein interactions such as CUT&RUN or those that measure chromatin accessibility, such as ATAC-seq.

(F) Single-particle tracking of proteins and chromatin loci has been the gold standard to measure their dynamics. New technological developments are overcoming old hurdles such as motion blur and photobleaching, greatly improving tracking.

(G) Mapping global chromatin dynamics is now possible using an optical flow-based technique called Hi-D. Here, thousands of trajectories are generated for a single nucleus, which in turn can be used to generate a heatmap of motion types, i.e., subdiffusive or directed motion. The next frontier in chromatin research will be to apply these technologies to organoids, tissues, and multicellular organisms (common as well as more unusual).

higher spatial resolving capabilities, electron microscopy (EM), which has been instrumental in the characterization of euchromatin and heterochromatin, cannot identify specific genomic regions (Monneron and Bernhard, 1969). In contrast, FISH and *in situ* sequencing-based approaches have the advantage of obtaining both the spatial and genomic information of the signal in single cells. Chromosomes were first directly revealed to be organized into distinct spatial territories in the cell nucleus during interphase by 3D-FISH probes designed to target-specific chromosomes (Lichter et al., 1988). However, these early FISH techniques did not have the genomic and spatial resolution necessary to characterize finer chromosome structures and the organization of chromatin at the sub-microscopic scale.

With the advent of chromosome conformation capture (3C)-based technologies and the discovery of novel structures such as topologically associated domains (TADs), compartments, and loops (Dekker et al., 2002; Gibcus et al., 2018; Rao et al., 2014), excitement has grown to understand how these structures manifest in single cells, and how they contribute to various genome functions. A major advance in FISH-based technology was the development of single-molecule FISH (smFISH), first demonstrated to detect single mRNA molecules as a diffraction-limited spot (Femino et al., 1998). Another catalyst was the adoption of massively parallel oligonucleotide synthesis methods to generate customized complex oligonucleotide libraries such as Oligopaint (Beliveau et al., 2012), which greatly facilitated the detection of multiple non-repetitive nucleic acid species.

The density of target materials in the cell nucleus has long posed a challenge for chromatin imaging. In recent years, the combination of super-resolution light microscopy and DNA FISH has emerged as an attractive method to study the physical organization of chromatin and revealed distinct organization principles between different epigenetic domains and their interfaces (Beliveau et al., 2015; Boettiger et al., 2016). However, in

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Table 1. Summary of Chromatin Technologies Discussed in This Review

Chromatin Technology	Application	Highlighted References
Imaging		
Multiplex FISH	chromosome structure and organization in living cells	Beliveau et al., 2012, 2015; Bintu et al., 2018; Boettiger et al., 2016; Chen et al., 2015; Guan et al., 2017; Liu et al., 2020; Lubeck et al., 2014; Mateo et al., 2019; Nir et al., 2018; Takei et al., 2017; Wang et al., 2016
Single-particle tracking	protein and DNA motion tracking	Gebhardt et al., 2013; Hansen et al., 2018; Lee et al., 2017; Liu et al., 2015; Mazza et al., 2012; Persson et al., 2013; Rhodes et al., 2017
Hi-D, DFCC, Deep-PALM, single nucleosome imaging	nucleosome, chromatin domain, and global chromatin dynamics, correlated motion	Barth et al., 2020a; Gupta et al., 2019; Voulodimos et al., 2018; Wang et al., 2019a; Wei et al., 2019; Weigert et al., 2018; Barth et al., 2020b; Hihara et al., 2012; Miron et al., 2019; Nagashima et al., 2019; Nozaki et al., 2017; Shaban and Seeber, 2020a, 2020b; Shaban et al., 2018, 2020
Computational imaging	resolve images using machine learning algorithm	Barth et al., 2020a; Gupta et al., 2019; Voulodimos et al., 2018; Wang et al., 2019a; Wei et al., 2019; Weigert et al., 2018
Chromatin Manipulation		
Optical tweezers	physical manipulation of chromatin with lasers	Bennink et al., 2001a; Kanger et al., 2008; Leuba et al., 2000; Marko and Siggia, 1995, 1997; Miroshnikova et al., 2017; Roopa and Shivashankar, 2006; Syrchina et al., 2020
Nuclear micromanipulation	study the physical properties of the nucleus such as force and elasticity	Banigan et al., 2017; Stephens et al., 2017, 2018, 2019a, 2019b
Genomics		
CUT&RUN, CUT&Tag, ssChiC-seq	DNA-protein interactions	Carter et al., 2019; Kaya-Okur et al., 2019; Ku et al., 2019; Nair et al., 2019; Skene and Henikoff, 2017
ATAC-seq	chromatin accessibility	Buenrostro et al., 2013, 2018; Cusanovich et al., 2015; Lareau et al., 2019; Preissl et al., 2018

order to map the 3D structure of large stretches of chromatin at higher genomic resolution, the detection and multiplexing capabilities of FISH labeling need enhancement. More recently, multiplexed FISH approaches that were developed for RNA imaging (Chen et al., 2015; Lubeck et al., 2014) were adapted to label tens of genomic regions corresponding to TADs observed by Hi-C in single human cells (Wang et al., 2016), and was later applied to mammalian tissues in combination with multiplexed RNA FISH (Liu et al., 2020). Improved multiplexed imaging combined with super-resolution stochastic optical reconstruction microscopy (STORM) revealed highly variable chromatin domains that form independent of cohesin (Bintu et al., 2018; Nir et al., 2018). This method has also been applied to developing *Drosophila* embryos in combination with RNA FISH to illustrate the relationship between enhancer-promoter interaction and nascent transcription (Mateo et al., 2019). Another approach is to combine multiplexed FISH labeling with live-cell chromatin imaging, where the identity of genomic regions of interest can be identified after their dynamics have been recorded in the same cell (Guan et al., 2017; Takei et al., 2017). With *in situ* imaging-based transcriptomics methods now capable of profiling more than 10,000 nucleic acid species (Eng et al., 2019; Shah et al., 2018; Xia et al., 2019a), it is intriguing to entertain the possibility of adapting them for chromatin tracing of whole chromosomes or even the entire genome at high resolution to study various open questions in genome biology in combination with RNA and protein imaging.

Imaging of Whole-Chromosome Dynamics on Macroscopic Length Scales in Living Cells

Chromosomes exhibit fascinating behaviors on length scales ranging from ~100 nm to multiple microns, at all stages of the mitotic cell cycle, and in meiosis. Such macroscopic effects are uniquely accessible by high-resolution fluorescence imaging of chromosomes in living cells, in two and three dimensions and, ideally, over time.

Macroscopic chromosomal behaviors are particularly apparent in the latter stages of the mitotic cell cycle, when

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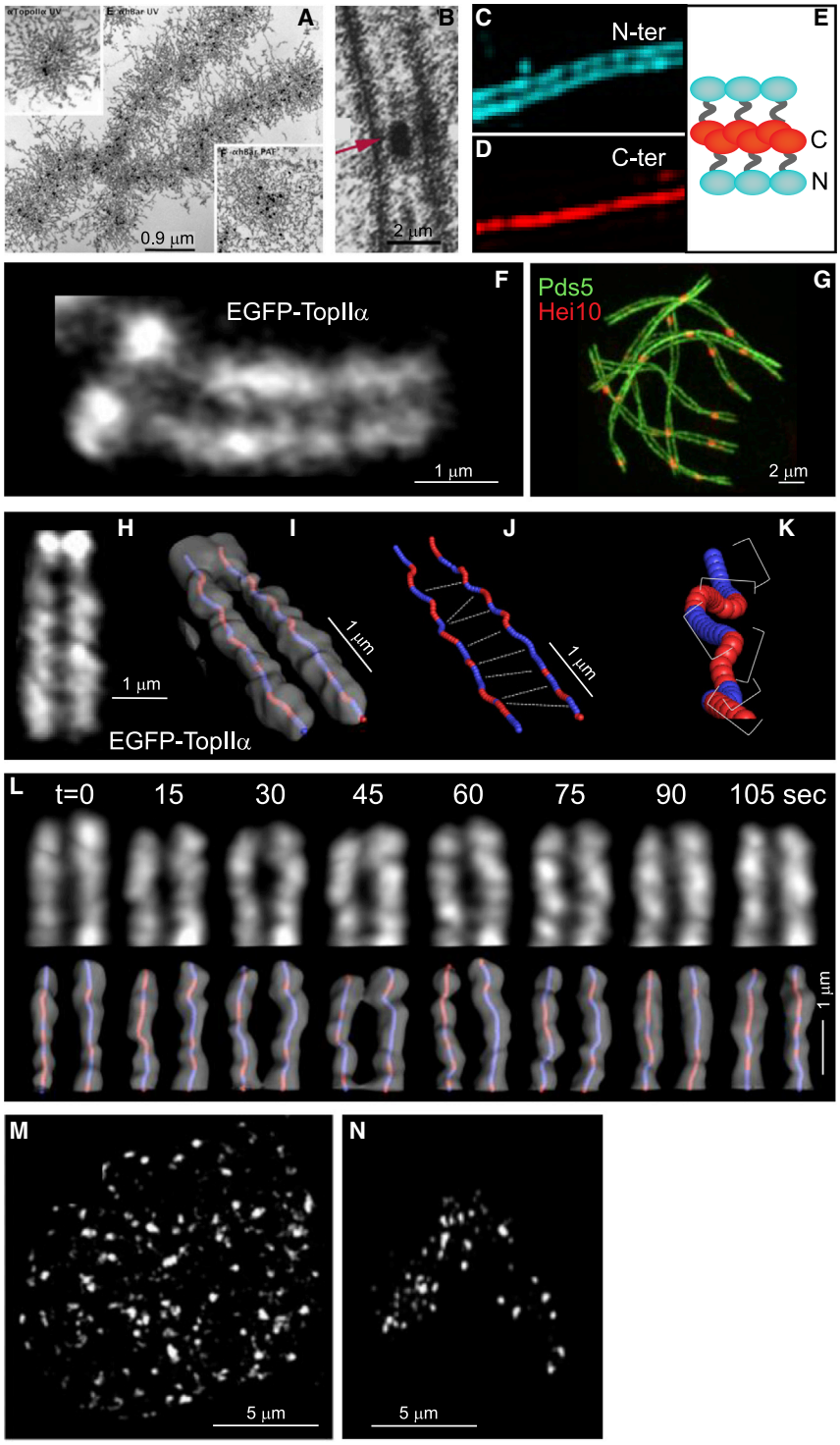

Figure 2. Whole-Chromosome Imaging

(A) EM images of an isolated mitotic chromosome (Maeshima et al., 2005).

(B) EM image of a 3D section showing a segment of synaptonemal complex (SC) with associated crossover recombination complex (arrow) (Zickler and Espagne, 2016).

(C–E) 3D-SIM images of the SC transverse filament protein GFP-tagged at the C terminus (C) or the N terminus (D) reveal molecular orientations of dimer complexes (E) (Dubois et al., 2019).

(F) 3D Airyscan snapshot of EGFP-TopIIα in a living cell reveals the bulky mitotic chromosome axis meshwork and linkage of sister axes by evenly spaced "mini-axis" bridges (Chu et al., 2020).

(G) 3D-SIM image of a nuclear complement of SCs with lateral elements are tagged by Pds5-GFP and (evenly spaced) crossover recombination complexes tagged by HEI10-mCherry (De Muyt et al., 2014).

(H–K) Mitotic chromosome axes and bridges visualized as in (F) (H) rendered in PyMOL along with corresponding axis intensity centroid paths of sister chromatids (I). Red and blue indicate left- and right-handed helicity, respectively. (J) Centroid paths in (I) with bridge positions indicated as defined from (H). (K) Enlarged segment of an axis centroid path showing sequential half-helical segments of alternating handedness (perversions).

(L) 3D time-lapse movie of TopIIa axes imaged and analyzed as in (H) and (I) (bridges not visible) showing dynamic fluctuations over <15 s timescale. (F and H–L are from Chu et al. [2020].)

(M and N) Muntjac (DM87) chromosomes labeled with Alexa555-dUTP in S-phase and imaged immediately after labeling (M) or, after segregation of a single chromosome, four generations later (N). Images taken with Nikon Ti wide-field epi-fluorescence microscope and filtered using a Two-stage Likelihood Pipeline (N. Vincenten, F. Chang, and N. Kleckner, personal communication).

chromosomes (and their sister chromatids) become discrete, individualized objects. Similarly, meiosis involves a complex dance of interactions between homologous chromosomes, which occurs during a prolonged prophase stage. Early light microscope visualizations were followed by EM, which affords nanometer resolution, e.g., of mitotic metaphase chromosomes (Figure 2A from Maeshima et al., 2005) and the meiotic synaptonemal complex (SC), a conserved structure that links homologous chromosome axes (Figure 2B from Zickler and Espagne, 2016). Super-resolution fluorescence imaging methodologies, plus immuno-gold EM, can now define the molecular composition of macroscopic features (e.g., as for SC transverse filaments [e.g., Figures 2C–2E from Dubois et al., 2019; Hernández-Hernández et al., 2016] and axes [Köhler et al., 2017]). In addition, super- and high-resolution fluorescence imaging can reveal global features that have previously been missed (e.g., mini-axis bridges between mitotic sister chromatids [Figure 2F from Chu et al. [2020], in this issue of *Molecular Cell*]).

Delineation of macroscopic features also reveals spatial patterning along chromosomes, which implies the presence of communication over long length scales. A classic example is meiotic "crossover interference" (Kleckner et al., 2004). Crossover recombination complexes occur at different positions in

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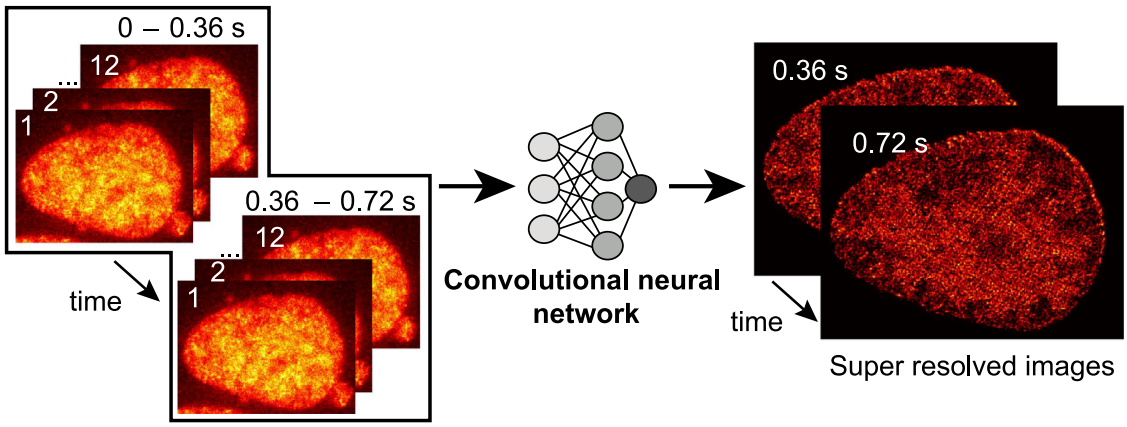

Figure 3. Deep-PALM Uses a Convolutional Neural Network to Super-Resolved Images
Photoactivated localization microscopy images of U2OS nuclei expressing H2B-PATagRFP are input to a trained deep convolutional neural network (CNN). The predictions from multiple input frames (30 ms/frame) are summed to construct a super-resolved image of chromatin *in vivo* with a final frame interval of 360 ms.

different nuclei; nonetheless, they always tend to be evenly spaced along the chromosomes (Figure 2G from De Muyt et al., 2014). Mitotic chromosomes also exhibit spatial patterns: even spacing of inter-sister bridges (Figure 2F) and axis paths comprising sequential half-helical segments of alternating helical handedness ("perversions") (Figures 2H–2K from Chu et al. [2020]). Understanding such spatial patterns, including determination of whether communication occurs by mechanical stress redistribution (Kleckner et al., 2004) and/or reaction-diffusion (e.g., Vecchiarelli et al., 2014), is an interesting area for future investigation.

The importance of visualizing chromosomes in living cells cannot be overstated. The potential for artifacts casts a shadow on all fixed-cell chromosome studies, a risk sometimes justified and sometimes not. Video micrographs of large plant chromosomes gave the first glimpse of the dynamics of whole individualized chromosomes in living cells (Bajer, 1965; Inoué and Oldenbourg, 1998). Modern fluorescence imaging of living cells removes the threat of fixation artifacts, with the added power of visualizing specific molecules, albeit with new limitations due to photobleaching and phototoxicity.

Most importantly, coupling of time-lapse imaging of living cell chromosomes with other approaches reveals new dynamic behaviors. For example, the process of chromosome compaction is seen to be accompanied by dynamic fluctuations in axis conformation on timescales of 15 s or less (Figure 2L from Chu et al. [2020]). Coupling of single-chromosome time-lapse imaging with degron analysis or optogenetic removal can reveal the consequences of molecular elimination on an individual chromosome basis, in real time, thereby complementing molecular and/or population studies. A holy grail would be the real-time detection of communication along chromosomes during spatial patterning. Overall, chromosomes are ultimately coherent mechanical objects whose evolution, motions, and behaviors reflect the operation of internal and external forces (Kleckner et al., 2004; Liang et al., 2015; Marko and Poirier, 2003). Such effects can only be investigated by visualization of individual chromosomes in living cells over time, in unperturbed cells or with genetic or physical modulation of mechanical perturbations.

Unique insights into whole-chromosome dynamics can also be provided by new applications of the well-known methodology in which fluorescent nucleotides are incorporated into chromosomes during S-phase (Manders et al., 1999; Schermelleh et al., 2001). Under appropriate conditions, such incorporation can create an array of chromosome speckles throughout the entire genome or, after several rounds of division, individual chromosomes (Figures 2M and 2N) with differentiation of AT- and GC-rich regions (Schermelleh et al., 2001) (and thus the A and B compartments revealed by Hi-C), in 3D over desired time intervals and time spans. Tracking of such signals in low signal-to-noise ratio regimes can define the dynamics of whole-chromosome behaviors throughout the cell cycle, before and after they emerge as discrete objects.

Chromosomes are the basis of heredity, both for single cells and for sexually reproducing organisms. The power of modern 3D time-lapse fluorescence imaging, with increasing capacity for detection at low signal-to-noise ratios, and coupled with experimental perturbations, promises to unleash a new wave of understanding from unique molecular, mechanistic, dynamic, and mechanical perspectives.

Studying Whole-Genome Organization and Dynamics in Living Cells Using Deep-PALM

As mentioned above, linking chromatin structure to function is one of the most important problems in chromatin biology. Fluorescent live-cell imaging is an ideal method to address this problem (Shaban and Seeber, 2020a). However, a number of technical hurdles must be addressed to study the dynamics and structure of the whole genome in living cells. First, a high-resolution live-cell imaging method is needed that can resolve chromatin structure at temporal resolutions necessary to capture dynamics. Second, an analysis method is required that can spatially resolve bulk and irregular chromatin motion over time with nanoscale sensitivity.

Deep-photoactivated localization microscopy (Deep-PALM) surmounts the first hurdle and simultaneously captures the structure and dynamics of chromatin at high temporal and

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sub-diffraction limited spatial resolutions (Barth et al., 2020a). Deep-PALM is a live-imaging technology employing a convolutional neural network (deep-learning algorithm, Figure 3) to predict super-resolution images from activated fluorophore-labeled histone proteins. By tuning the network to experimental conditions, a time resolution of 360 ms at a spatial resolution of 65 nm could be achieved, enabling Deep-PALM to resolve elongated (~45 to 90 nm wide) chromatin nanodomains (blobs). Each blob comprises a number of associating nucleosomes (<30), which assemble transiently over the timescale of about 1 s. Estimating the structural parameters such as nearest neighbor distance and size of chromatin blobs show that those blobs are consistent with structures identified in single-molecule localization imaging in fixed cells (Ricci et al., 2015; Xu et al., 2018). The dynamic properties of these blobs are in line with other work based on structural illumination super-resolution microscopy (Miron et al., 2019). To connect the blob formation to biological function, first it would be important to address if blobs consist of the same monomers or if blobs are random formations like a by-product of other processes (activity and polymer topology for instance), intermediate stage, or direct outcome of some biological process (Barth et al., 2020b).

Recently, a set of two methods overcame the second hurdle to track bulk chromatin motion with sub-pixel accuracy (nanoscale resolution) in living cells (Shaban et al., 2018, 2020). Based on the combination of light microscopy and computer vision (Optical Flow) technology, the methods reconstruct the dynamics of bulk chromatin in diffraction-limited optical microscopy images at nanoscale resolution throughout the entire nucleus simultaneously. The first method, called Dense Flow reconstruction and Correlation (DFCC), characterizes and quantifies spatially correlated motion of chromatin (Shaban et al., 2018). The complementary second method, high-resolution diffusion mapping (Hi-D), uses Bayesian inference to relate the observed dynamics pixel by pixel to diffusion models, providing insights into the underlying physics of chromatin dynamics (Shaban et al., 2020). Hi-D builds two-dimensional, high-resolution maps of biophysical properties of the entire nucleus for an integrated characterization of diffusion processes acting on the chromatin fiber at the local and global scale. A combination of Deep-PALM and Hi-D was applied to quantitatively analyze chromatin blob dynamics at nanoscale sensitivity (13.5 nm reconstructed pixel size) and couple it to structural parameters (Barth et al., 2020a).

This technology could be implemented in future work to answer questions on how chromatin structure and dynamics control gene regulation (Shaban and Seeber, 2020a), DNA repair (Shaban and Seeber, 2020b), replication, and genome organization. With regards to transcription, this technology will be able to test whether chromatin undergoes structural reorganization when shifting from inactive to actively transcribing states and how epigenetic modifications may alter this process. In summary, deep-learning and computer vision methods in combination with optical microscopy pave the way to answering the thorniest questions in chromatin biology.

Single-Particle Tracking of Nuclear Proteins

While studying the dynamics of chromatin is important to understand processes such as transcription, equally important is the need to examine the dynamics of nuclear proteins including transcription factors (TFs). Single-particle tracking (SPT, or single-molecule tracking) is an attractive technology to do so, since it allows direct visualization of the protein of interest (POI) in its native environment inside the cell. Moreover, SPT can provide nanometer resolution in space and millisecond resolution in time. Recent advances in protein labeling technology, fluorescent dyes, microscopy, and computational analyses now make SPT of nuclear proteins a much more widely accessible technology (Liu et al., 2015). While a wide range of distinct SPT approaches have been reported, the basic principles are the same (Shao et al., 2018). First, the POI is fluorescently labeled, typically with a genetically encoded fluorescent tag, including classic fluorophores, photoswitchable fluorophores (for example, mEos) or with a self-labeling tag such as HaloTag. Second, single proteins are imaged using microscopy techniques that minimize background noise such as total internal reflection fluorescence (TIRF) or highly inclined thin illumination (HILO) (Tokunaga et al., 2008). Third, molecules are localized in each frame and then linked between frames to form trajectories (Lee et al., 2017). Fourth, using the example of DNA-binding proteins such as TFs, the trajectories are analyzed to extract a multitude of information including: the fraction that is bound to DNA, the diffusion coefficients of the bound and free states, the residence time of DNA binding, nuclear organization and clustering, anomalous diffusion, target search mechanism, etc. (Gebhardt et al., 2013; Hansen et al., 2018; Lee et al., 2017; Mazza et al., 2012; Persson et al., 2013). These SPT steps are illustrated in Figure 4, using stroboscopic photo-activation SPT (spaSPT) (Hansen et al., 2018), which relies on photo-activatable Janelia Fluor dyes to minimize tracking errors (Grimm et al., 2016; Manley et al., 2008) (Figure 4A) and on stroboscopic excitation to minimize "motion-blur" artifacts (Elf et al., 2007; Hansen et al., 2018) (Figure 4B).

These technological advances have led to a number of new biological insights: SPT has been instrumental in probing how DNA-binding proteins search for and find their target sites inside the crowded nucleus (Hansen et al., 2020; Izeddin et al., 2014; Rhodes et al., 2017). Work from many laboratories suggest that transcription complexes bound to chromatin are unstable with residence times of seconds to tens of seconds (Gebhardt et al., 2013; Mazza et al., 2012; Mir et al., 2018; Shao et al., 2018; Swinstead et al., 2016; Teves et al., 2016). Related to this, SPT has also been used to study the function, dynamics, and selectivity of higher order clusters or hubs of TFs and RNA Polymerase II, as well as how hub formations can be mediated by intrinsically disordered or low-complexity protein domains (Boehning et al., 2018; Cho et al., 2018; Chong et al., 2018; Lu et al., 2018; McSwiggen et al., 2019). SPT has also provided insight into chromosome structure; for instance, SPT studies of CTCF and cohesin, the proteins that form chromosomal loops, suggest that these loops are likely to be dynamic (Hansen et al., 2017) and that only a subset of nuclear proteins serve as mitotic bookmarkers (Oomen et al., 2019; Raccaud et al., 2019; Teves et al., 2016). Importantly, SPT has also been used to probe the function and stoichiometry of the Polycomb complex *in vivo*, revealing for example how the oncohistone H3.3K27M dysregulates this complex (Tatavosian et al., 2018;

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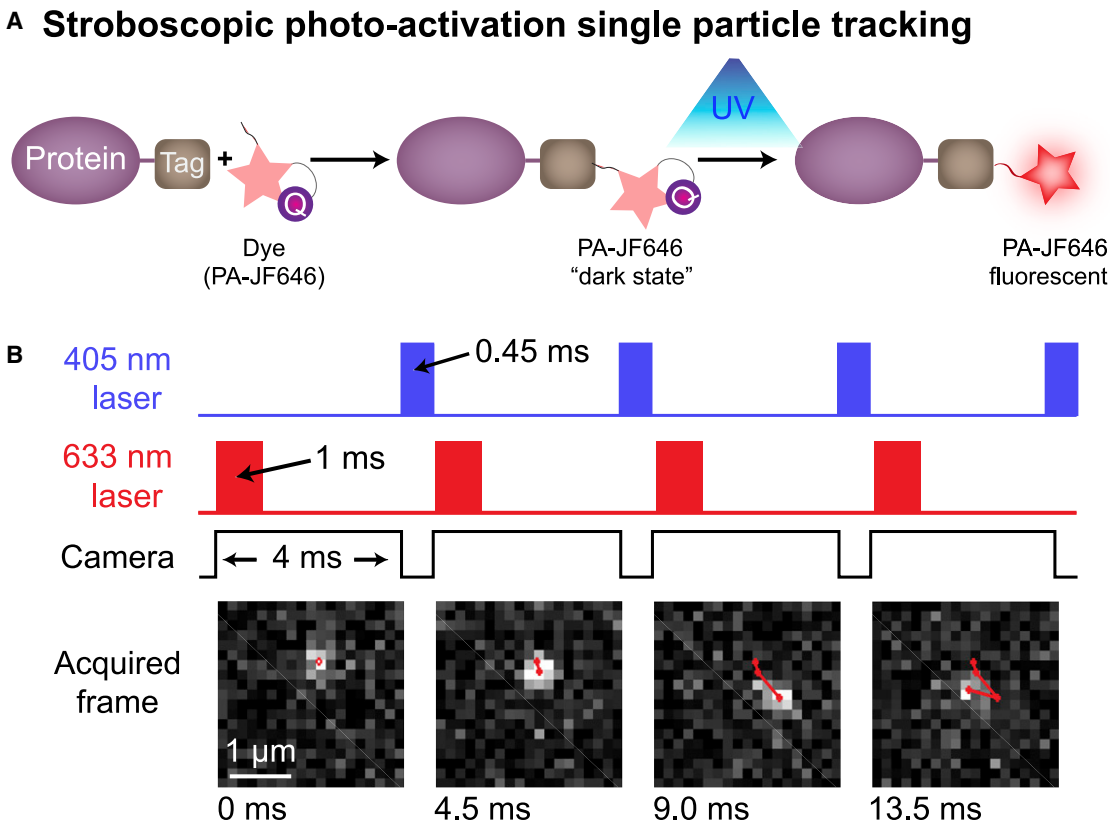

Figure 4. Stroboscopic Photo-activation Single-Particle Tracking

(A) Protein of interest is labeled with a small tag, e.g., Halo- or SNAP-Tag, which binds to a fluorescent dye in its dark state. Upon UV activation, the dye becomes fluorescent and is imaged on a TIRF microscope. Molecules are localized in each frame and linked to form trajectories.

(B) By using pulsed laser excitation, "motion-blur" bias, which results from the protein moving while the image is acquired, can be minimized. Using photo-activation, the number of fluorescent proteins can be kept low to minimize tracking errors. Adapted and reproduced with permission from Hansen et al. (2017).

Youmans et al., 2018). In summary, SPT of nuclear proteins has recently grown in popularity due to technological advances. This approach is revealing many new biological insights and its growth shows no signs of slowing down.

Single-Nucleosome, Live-Cell Imaging

Recent evidence suggests that there is, in general, no static 30 nm chromatin fiber in living cells (Eltsov et al., 2008; Fussner et al., 2012; Nishino et al., 2012; Ou et al., 2017). Rather, other chromatin models have been proposed that highlight the irregular folding of chromatin into domains that resemble liquid droplets as well as its dynamic nature (Maeshima et al., 2010). Chromatin labeling, developed in the 1990s, is widely used and can be classified into two types: sequence-specific and non-specific labeling. Genetically encoded bacterial systems such as LacO/LacI or the ANCHOR (INT/ParB) systems, engineered DNA binding proteins such as TALE-, or CRISPR/Cas9-based labeling systems rely on sequence specificity and can visualize specific genomic regions (reviewed in Seeber et al., 2018). Alternatively, chromatin can be labeled in bulk, for instance, through pulse labeling of DNA replication domains with fluorescent nucleotides, live-cell DNA labels such as Hoescht conjugates or fluorescently tagged histone proteins as mentioned in the previous sections (Bucevičius et al., 2018; Jackson and Pombo, 1998; Manders et al., 1999; Markaki et al., 2010; Nozaki et al., 2017; Schermelleh et al., 2001; Xiang et al., 2018). Replication domains have an average diameter of approximately 110–150 nm and, while they cannot be stained specifically, they can be differentiated into eu- or heterochromatin domains by timed labeling (Schermelleh et al., 2001) that revealed differences between the dynamics of euchromatin and heterochromatin (Nozaki et al., 2017).

Although single-nucleosome labeling is not currently sequence specific, it does enable us to label and observe the motion of nucleosomes in a whole nucleus. Based on the principles of single-particle tracking PALM (sptPALM) (Betzig et al., 2006; Manley et al., 2008), a number of nucleosomes are activated and tracked over time (e.g., ~100 H2B molecules/time frame (50 ms/nucleus) (Nagashima et al., 2019; Nozaki et al., 2017). These experiments show that nucleosome movement is sub-diffusive and likely constrained by linker DNA (Hihara et al., 2012; Nagashima et al., 2019; Nozaki et al., 2017). By plotting the magnitude of nucleosome dynamics as a 2D heatmap, the spatial distribution of nucleosome dynamics has been visualized in an entire live nucleus (Nagashima et al., 2019; Nozaki et al., 2017). This heatmap revealed the non-homogeneous distribution of nucleosome dynamics: the interior region of the nucleus enriched with euchromatin showed higher movement of nucleosomes, whereas the periphery of the nucleus or the

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nucleolus enriched with heterochromatin showed lower movement. Combined with correlative immunostaining of proteins, nucleosome movement around specific proteins could also be assessed. Heterochromatin marker trimethylation of histone H3 Lys9 (H3K9me3) indicated reduced nucleosome dynamics around the heterochromatin region (Nozaki et al., 2017). Furthermore, single-nucleosome imaging is easily dealt with a polymer model or statistical analysis and could retrieve more information from single-molecule live-cell imaging data that we could not obtain from fixed cells (Ashwin et al., 2019; Hihara et al., 2012; Maeshima et al., 2015; Shinkai et al., 2016). However, the relationship between chromatin and nucleosome dynamics revealed by chromatin or nucleosome labeling remains obscure.

It is possible to use single-nucleosome imaging based on H2B-PAmCherry to generate super-resolution images of chromatin using sptPALM. Nucleosome distributions displayed clustered patterning, supporting the notion of chromatin domain formation with diameters of approximately 220 nm in living cells (160 nm in fixed cells), (Nozaki et al., 2017). An important question to answer is whether domain movement reflects individual nucleosome movement? To address this question, dual-color labeling and imaging of the single-nucleosome and DNA replication domains was performed. This imaging showed that nucleosomes and nearby domains (<150 nm) moved correlatively, whereas those localizing far apart from one another (>150 nm) moved independently. This suggests that domain movement reflects local nucleosome movement. A more speculative indication from these data is that nucleosomes form a chromatin domain composed of condensed structures like ''liquid droplets'' rather than loose bundles of fibers or extended loops (Nozaki et al., 2017).

In conclusion, chromatin and single-nucleosome live-cell imaging will continue to contribute to change the view of chromatin from fixed and static to irregular and dynamic nature (e.g., liquid droplets of chromatin (Gibson et al., 2019; Maeshima et al., 2010). The loop extrusion model, proposed mainly based on Hi-C data (Fudenberg et al., 2017) will eventually be tested in living cells. Combined with single-molecule imaging of nuclear proteins as described above, live-cell imaging will shed light on how chromatin affects physiological function including nuclear protein target search and the relationships between chromatin features, epigenetic marks, and DNA transactions.

Tools to Manipulate Chromatin and the Nucleus in Living Cells

Measuring the Mechanical Properties of Chromatin with Optical Tweezers

Double-stranded DNA is stiff, as revealed by experiments and modeling of force-extension curves where the free end of the rod-like DNA polymer is pulled by a magnetic bead, while its other end is tethered to a surface (Marko and Siggia, 1995). However, chromatin being an assembly of DNA and histone proteins provides a more complex mechano-rheological microenvironment within the nucleus, the functional consequences of which are only beginning to be understood.

Cells are constantly subjected to mechanical cues such as shear stress, differential tissue rigidity, or osmotic stresses, which could be transmitted to the subnuclear lamina via the cytoskeleton. Within the nucleus, chromatin being an active mechanical component, is capable of undergoing dynamic changes due to the application of stresses, which could then have consequences on mechanotransduction of chromatin mediated responses and thence gene expression (Miroshnikova et al., 2017). With the advent of tools such as optical tweezers, it became possible to manipulate small microscopic objects with nanometer precision as well as control and exert forces in the range of 0.1–100 pN. Using optical tweezers and microfluidic flow cells, the force required to unwrap DNA from the histone octamer could be measured (20 pN-40 pN), providing a direct way to study the energetics of the chromatin fiber assembly (Bennink et al., 2001a; Brower-Toland et al., 2002). Later work used optical tweezers to study remodeling of chromatin fibers and their viscoelasticity by disrupting the tail-tail interactions via enzymes, which could directly be captured via the decrease in stiffness of the chromatin by sensitive force measurements (Roopa and Shivashankar, 2006). While several studies (Bennink et al., 2001b; Kanger et al., 2008; Leuba et al., 2000; Marko and Siggia, 1997; Pope et al., 2002) have successfully unraveled the mechanical properties of both DNA and chromatin fibers *ex vivo*, the real challenge is to connect the dynamics of the chromatin associated with applied stresses in physiological conditions *in vivo*. Given the optical contrast between euchromatin and heterochromatin within the nucleus, optical tweezers are a promising tool to probe mechanical properties such as stiffness and viscous dissipation by applying controlled amounts of forces to the different regions of chromatin. Phagocytosed polystyrene beads with appropriate chemical modifications could also be used to tether parts of chromatin, which could then be manipulated using the tweezers (Figure 1). To study functionally relevant scenarios, one could mimic natural biochemical changes such as acetylation or other modifications to the chromatin microenvironment and directly study the changes caused in the mechanical properties of the chromatin. Using live markers to tag reporters of gene expression of domains that are suspected to be active in mechanotransduction, actively stretching chromatin could unravel the role and threshold forces required to induce expression. Optical tweezers not only provide a way to capture the properties of chromatin in endogenous settings within the nucleus but also allow active manipulation of the nuclear microenvironment to modulate biological functions such as gene expression (Bracha et al., 2019).

Nuclear Micromanipulation to Study Chromatin Mechanics

Micromanipulation allows for gentle isolation of a single nucleus from living cells to do force measurement, biochemical, and imaging studies not possible with other technologies. This novel technique was adapted from micromanipulation studies of single mitotic chromosomes, which detailed the roles of DNA and chromatin proteins in chromosome compaction, organization, and mechanics (Kawamura et al., 2010; Poirier et al., 2002). Using micromanipulation of micropipettes a single nucleus can be isolated via local spray lysis of the cell and recovered via slight aspiration and non-specific attachment of micropipettes at opposite ends (Stephens et al., 2017). Force-extension measurements of the cell nucleus are accomplished by a ''pull'' pipette extending the nucleus (ΔL), while the ''force'' pipette's deflection (Δx),

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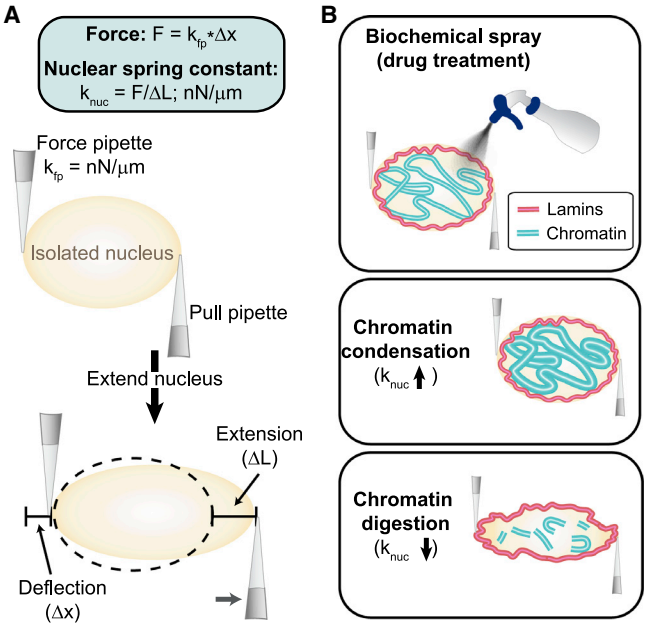

Figure 5. Nuclear Micromanipulation

(A) Micromanipulation force measurement schematic of how the technique works. A force pipette (fp) and a pull pipette (pp) are used to extend the nucleus (nuc).

(B) A single isolated nucleus can be measured before and after biochemical treatment, providing fine-tuned control over nuclear chromatin mechanical and imaging measurements. The nuclear spring constant (k_{nuc}) increases during chromatin condensation and decreases after chromatin digestion.

multiplied by a pre-measured bending constant (k_{fp}), provides a measure of force (Δx*k = F, Hookes' law; See Figure 5A). Force-extension ultimately provides a measurement of the nuclear spring constant (k_{nuc} = F/ΔL; nN/μm). This technique allows for a wide range and fine control of both speed and length of extension providing the ability to explore both elastic and viscoelastic regimes. Biochemical treatments added via a third spray micropipette in conjunction with reproducible elastic force-extension measurements allow for unparalleled assaying of the same isolated nucleus before and after treatment (Figure 5B). This treatment can include antibody labeling (Biggs et al., 2019; Sun et al., 2018) or fluorescently tagged protein (Banigan et al., 2017) and microscope imaging of structures before, during, and/or after physical manipulation and/or biochemical treatments.

Single-nucleus micromanipulation force measurements have provided, for the first time, the ability to separate the different mechanical contributions of chromatin (short extensions) from lamins (strain stiffening at long extensions) to the cell nucleus (Stephens et al., 2017). This technique has led to findings showing that decreased chromatin-based nuclear stiffness modulated by histone modification state results in abnormal nuclear morphology, a hallmark of human disease (Stephens et al., 2018). Abnormal nuclear mechanics and morphology causes rupturing of the cell nucleus leading to nuclear dysfunction such as increased DNA damage (Pfeifer et al., 2018; Stephens et al., 2019a; Xia et al., 2019b). Function can be rescued via increased chromatin-based nuclear mechanics via heterochromatin formation through a mechanotransduction pathway (Stephens et al., 2019b). Biochemical treatments in conjunction with force-extension measurements revealed that, upon DNA digestion, the short extension stiffness regime of the nucleus was lost (Stephens et al., 2017) and the morphology of the nucleus was compromised (Banigan et al., 2017). These data solidify chromatin's role as a major nuclear mechanical component dictating nuclear mechanics, morphology, and function.

Micromanipulation-based techniques provide a depth of single-cell measurements that could be more largely explored by cell biologists. An easy way to access this technique is to use patch-clamp experimental setups that include the ability to make micropipettes and control them via micromanipulators. Micromanipulation is being used as a parallel technique with Hi-C to assay chromatin interaction frequency via mechanical strength maintenance. Recent studies using various degrees of DNA digestion revealed the interaction frequency of the genome to be 10–25 kb as measured by physical resistance (micromanipulation) and proximity (Hi-C) (Belaghzal et al., 2019). Beyond force measurements, isolation of single nuclei via micromanipulation could be combined with other single-cell studies, *in vitro* biochemistry via micropipette spray, and imaging to provide experimental capabilities not previously possible. Micromanipulation setups can be fit onto the microscope of your choice, meaning super-resolution imaging is possible. Thus, we highly encourage chromatin and nuclear biologists to consider using micromanipulation as a new experimental approach for their research.

Chromatin-Protein Interactions

DNA-Protein Interactions at Single-Cell Resolution

Chromatin structure and gene expression are regulated by the combinatorial binding of chromatin-associated regulators, including a plethora of TFs, chromatin modifiers, histone modifications, and non-coding RNAs. Traditional genomics approaches to study these interactions have significantly enhanced our understanding of the interplay between these regulatory factors. However, these bulk measurements depend on population-averaged signals limiting their utility, providing little insight into several critical questions in epigenomics. For example, does heterogeneity in chromatin structure across a relatively homogeneous population of cells result in phenotypic heterogeneity? Is heterogeneity in gene expression (gene expression noise) regulated by chromatin structure? And how and when do cells commit to distinct lineages during the course of differentiation?

Adaptations to chromatin immunoprecipitation (ChIP) and DNA adenine methyltransferase identification (DamID) methods have enabled single-cell analysis of DNA-protein interactions (Kind et al., 2015; Rotem et al., 2015). These pioneering papers have motivated additional methods in the field and significantly intensified our interests in the gene regulatory mechanisms that operate at the single-cell level—often used to provide new insights into the molecular basis of lineage commitment. However, many of these methods suffer from low coverage arising from multi-step molecular biology assays or chromatin purification protocols.

Excitingly, new methods to profile DNA-protein interactions have emerged. For example, CUT&RUN (Figure 6A) and single-

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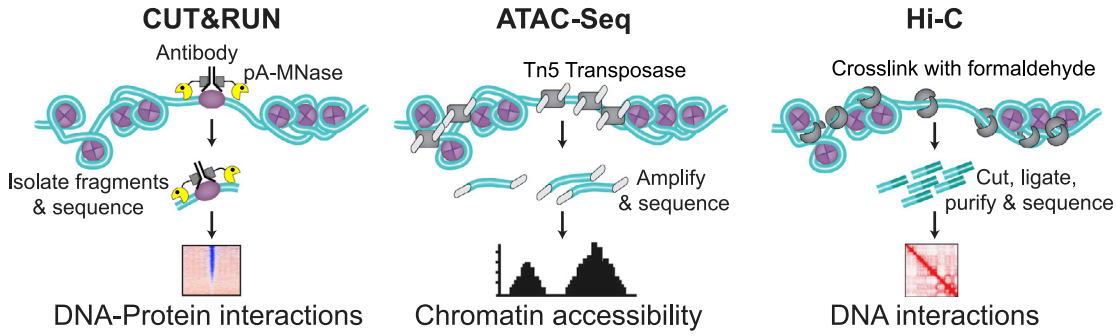

Figure 6. Probing Chromatin Using Sequencing-Based Genomic Approaches

(A) Cleavage Under Targets and Release Using Nuclease (CUT&RUN) uses a chimeric fusion of Protein A and MNase (pA-MNase). As protein A binds specifically to Immunoglobulin G of antibodies, this fusion can be used to target pA-MNase to any nuclear protein, such as a transcription factor, with a sufficiently specific antibody. MNase, activated by the addition of calcium, cleaves DNA, and the resulting fragments can be isolated and sequenced to determine DNA-protein interactions.

(B) Assay for transposase-accessible chromatin using sequencing (ATAC-seq) measures the accessibility of chromatin genome-wide by using Tn5 transposase to cleave DNA and integrate sequencing adaptors specifically into open chromatin. The resulting adaptor tagged DNA fragments are then amplified and sequenced to generate an ATAC-seq peak plot.

(C) While not discussed in detail in this review, Hi-C is a fundamental technique used to determine chromatin organization. In brief, chromatin is cross-linked using formaldehyde. Following this, chromatin is cut, adjacent pieces are ligated, and fragments are purified and sequenced. A contact map of DNA interactions can then be generated.

cell chromatin immunocleavage sequencing (scChIC-seq) fuses MNase to an antibody enabling low input or single-cell measurements (Ku et al., 2019; Skene and Henikoff, 2017). Further, several adaptations to this basic principle have been developed that make use of Tn5 transposase to probe-specific DNA-protein interactions. Cleavage Under Targets and Tagmentation (CUT&Tag) uses a proteinA-Tn5 fusion protein to target a specific antibody bound to the POI inside the nucleus. Tn5 is then activated to insert sequencing adapters in targeted chromatin sites (Kaya-Okur et al., 2019). Others have developed a similar approach (Carter et al., 2019) and further adapted it to combinatorial indexing (Nair et al., 2019). These methods have several advantages over previous methods, including the straightforward compatibility with current single-cell assay for transposase-accessible chromatin using sequencing (ATAC-seq) methods. As such, we suspect these tools to be adaptable to single-cell ATAC sequencing (scATAC-seq) droplet microfluidics platforms. We can, therefore, anticipate further development and adaptation of these methods to enhance our understanding of epigenomic heterogeneity at single-cell and a single-gene resolution.

There has been a significant interest to develop single-cell technologies offering a range of measurements from multiple angles of chromatin regulation. However, new computational tools and, importantly, continued improvement to our understanding of gene regulation will be essential for our understanding of how these distinct measurements reflecting different layers of gene regulation relate to one another. Further, we foresee an integration of epigenomic tools beyond these sequencing-based methods. Advanced imaging technologies (discussed in this review) provide a direct physical representation of the multilayered 3D chromatin structure. For example, recent developments in high-throughput oligo-based and multiplexed immunolabeling methods have blurred the lines between genomics and imaging, providing new opportunities for epigenomics (Chen et al., 2016; Goltsev et al., 2018; Saka et al., 2019). Furthermore, efforts have integrated imaging approaches with single-cell genome-wide chromatin conformation capture technologies (Stevens et al., 2017; Tan et al., 2018; Wang et al., 2016). One would imagine the development of an ideal technology that can integrate all these approaches to provide a highly resolved spatiotemporal map of epigenomic and transcriptomic events building a stepping stone to understand the complex developmental program of an organism discussed in the next section.

scATAC-Seq

Single-cell tools are already vastly improving our ability to measure epigenomic variability resulting from changes in chromatin accessibility. Classic studies have shown that nucleosome displacement or reorganization can lead to accessible chromatin. Assay for transposase-accessible chromatin using sequencing (ATAC-seq) makes use of Tn5 transposase to integrate sequencing adaptors into accessible (also known as "open") chromatin across the genome (Buenrostro et al., 2013) (Figure 6B). Methods using plate-based DNA barcoding and/or microfluidics with ATAC-seq have resulted in a repertoire of tools that enabled analysis of chromatin accessibility at single-cell resolution (scATAC-seq) (Buenrostro et al., 2018; Cusanovich et al., 2015; Lareau et al., 2019). Applications of these tools have demonstrated the utility of a single-cell approach by unmasking cellular heterogeneity and chromatin regulators of cell-fate determination during various biological processes, including differentiation, development, and embryogenesis (Buenrostro et al., 2018; Preissl et al., 2018).

While these methods are successful in identifying epigenetic states, there is a growing interest to integrate these measurements with the transcriptome within the same single cell to better understand the chromatin mechanisms leading to gene-expression change. As such, an increasing number of "multi-omic" methods (methods that seek to make multiple

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measurements within the same single cell) are emerging to bridge this technological gap (reviewed in Kelsey et al., 2017 and Shema et al., 2019) (Chen et al., 2019; Kelsey et al., 2017; Shema et al., 2019; Zhu et al., 2019). However, current multi-omic approaches are often limited by either scalability or sensitivity, making it difficult to resolve the temporal relationship between the epigenomics and the transcriptomic. Therefore, further improvements to current methods may provide an opportunity to infer regulatory relationships between chromatin change and gene expression outcomes to better understand the relationship between the epigenome and transcriptome.

New Concepts for Chromatin Labeling and Imaging

The Role of Phase Separation in Chromatin Organization

Euchromatin and heterochromatin separate into distinct domains in the cell nucleus. Recent evidence suggests that phase separation can drive the formation of heterochromatin domains (Gibson et al., 2019; Larson et al., 2017; Strom et al., 2017). A model emerges in which post-translational histone modifications are at the very heart of this physicochemical process. A characteristic feature of heterochromatin is H3K9 methylation bound by heterochromatin protein 1 (HP1). Both, *Drosophila* HP1a (Strom et al., 2017) and human HP1a (Larson et al., 2017) can oligomerize and support the formation of phase separated constitutive heterochromatin domains (Wang et al., 2019b). However, biophysical properties of HP1a *in vivo* support a collapsed polymer globule model rather than liquid-liquid phase separation of proteins driving chromocenter formation (Erdel et al., 2020). Phase separation of the Polycomb protein CBX2 has been implicated in the formation of facultative heterochromatin condensates (Tatavosian et al., 2019). Such biomolecular condensates can mechanically interact with chromatin. HP1 droplets can pull in and densely pack DNA (Larson et al., 2017), while other protein droplets have been shown to physically exclude chromatin (Shin et al., 2019). The heterochromatin phase presents a diffusion barrier permissible to some proteins but excluding others, akin to the phenylalanine-glycine hydrogel barrier in the core of nuclear pore complexes that only permits passage of macromolecular cargo decorated with transport receptors that interact with the hydrogel (Schmidt and Görlich, 2016). The linker histone H1 itself appears to promote phase separation of chromatin (Shakya et al., 2020). Nucleosome spacing as well as post-translational modifications confer specificity to condensates (Gibson et al., 2019). Chromatin with acetylated histones partitioned into distinct condensates when bound by multi-bromodomain proteins *in vitro* and when injected into live HeLa cells.

Chromatin transactions, such as DNA replication (Parker et al., 2019) and DNA damage repair (Kilic et al., 2019), have been reported to involve the chromatin-assisted formation of phase-separated nuclear bodies. In transcription, extended regulatory chromatin domains termed super enhancers control the expression of lineage-specific TFs (Whyte et al., 2013). These chromatin domains are characterized by extended H3K27 acetylation marks and binding of, among other factors, Mediator, RNA Polymerase II (Pol II), and the multi-bromodomain protein BRD4, all of which associate in condensates in mouse embryonic stem cells (Cho et al., 2018; Sabari et al., 2018). Inhibition of BRD4 binding to H3K27ac led to dissolution of condensates. Condensate-forming proteins often carry intrinsically disordered regions that can form multivalent interactions (Hyman et al., 2014), and non-coding RNA has been implicated in the formation of paraspeckles (Yamazaki et al., 2018), condensates at enhancers (Nair et al., 2019), DNA-damage-response foci (Pessina et al., 2019), and other nuclear bodies (Banani et al., 2017). Phosphorylation of the C-terminal domain (CTD) drives Pol II out of transcription condensates and leads to partitioning into splicing condensates (nuclear speckles) associated with highly transcribed chromatin domains (Chen et al., 2018b; Guo et al., 2019).

From a functional viewpoint, phase separation as a mechanism to organize chromatin and chromatin-based transactions provides a number of benefits (reviewed in Alberti et al., 2019; Banani et al., 2017; Hyman et al., 2014): (1) phase separation is a self-organizing process that does not require energy consumption, (2) the weak but multivalent interactions involved are easier to modulate than protein-protein interactions, (3) small changes in environmental conditions such as pH or temperature can elicit a strong response in systems near phase boundaries, (5) the formation of distinct types of immiscible condensates offers a mechanism to sequester molecules at high concentrations, and (6) partitioning of chromatin and macromolecules into condensates based on reversible marks (post-translational modifications, TF binding, etc.) can confer specificity in the crowded nuclear environment.

While liquid-liquid phase separation of low-order systems in thermodynamic equilibrium may be appropriate to describe droplet formation *in vivo*, these models do not generally apply to condensates in live cells (Berry et al., 2018; Erdel and Rippe, 2018; Söding et al., 2020). Condensates can mature from liquid to gel-like or solid states (Nair et al., 2019; Patel et al., 2015), modulation of ATP concentration or ionic strength tunes phase behavior (Wright et al., 2019), and condensates can consist of multiple phases themselves (Sawyer et al., 2019). Enzymatic activity by histone acetyltransferases, kinases, chromatin remodelers, or other ATP-consuming proteins can transform condensates into catalytically active droplets (Portz and Shorter, 2019) of dynamic composition. The experimental assessment of *in vivo* phase separation is further complicated by the small, diffraction-limited size of some phase domains and requires a range of complementary approaches (Peng and Weber, 2019; Alberti et al., 2019). The experimental methods and computational analyses outlined in this review combined with *in vitro* reconstitution under physiological conditions and theoretical and *in silico* molecular modeling will dissect nucleation and grow mechanisms, as well as structural roles of scaffold and client factors (Banani et al., 2016). This will ultimately help us understand the exact biophysical mechanisms driving phase separation and its regulation in the cell nucleus.

Dynamics and Organization of Nuclear Proteins Governed by Weak Transient Interactions

DNA-binding proteins interact transiently with many chromatin sites and specifically with their cognate binding sites (Halford and Marko, 2004). These interactions govern protein dynamics (Hansen et al., 2017; Misteli, 2001) and their distribution in the nucleus (Woringer and Darzacq, 2018). The wealth of

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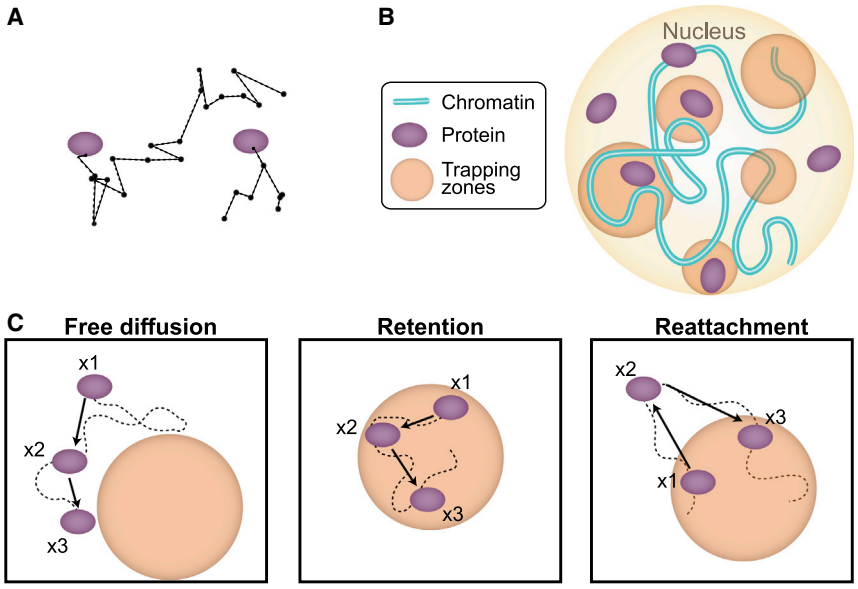

Figure 7. A Model for Nuclear Protein Dynamics Based on Retention and Reattachment

(A) Characteristic stochastic trajectories of tagged proteins.

(B) A model wherein protein diffusion in the nucleus is governed by its interaction with trapping zones within which there is a high concentration of binding sites. These zones could be composed of DNA, RNA, or a phase-separated condensate of protein.

(C) The protein (light blue) is observed at three time points (×1, ×2, ×3) along its stochastic trajectory (red curve). Left: outside of the zones, the protein performs free diffusion. Middle: while inside a zone, a protein can be reflected from its boundary. This would result in local retention. Right: if a protein leaves a zone, it is more likely to reattach to it, which would result in apparent anisotropy and sub-diffusion.

microscopy data generated studying the dynamics of nuclear proteins presents a major challenge for computational biologists: how to derive the rules of protein dynamics and distributions from chromatin configuration? Can we infer these interactions and chromatin structure by following the trajectories of tagged proteins (Figure 7A), or observing the manner in which they are distributed in the nucleus? To do so, a theory of protein dynamics and its interaction with chromatin is needed. Facilitated diffusion models (Bauer and Metzler, 2012), previously applied to prokaryotes (Hammar et al., 2012), suggested that proteins perform a mixture of two motion types – 1d sliding along the DNA and diffusion in 3d that allows for jumping between sites. Unlike bacterial DNA, mammalian DNA is chromatinized, which could rule out 1d sliding on DNA. While *in vitro* mammalian TF factors have been shown to slide along the DNA (Chen et al., 2014; McKinney et al., 2004), it is unclear whether this behavior would be dominant in the enormous mammalian nucleus. Chromatin, RNA, and proteins all appear to perform a motion with a curious phenomenology. The motion is often subdiffusive (Bancaud et al., 2009; Lampo et al., 2017; Shinkai et al., 2016; Weiss et al., 2004), where the mean squared displacement grows as a power law of time with an exponent smaller than one. In addition, the motion is often anisotropic, with a tendency for a backward step (Izeddin et al., 2014). These two features of motion could, in principle, fall into the fractional Brownian motion (Metzler et al., 2014), where this phenomenology is attributed to the memory response of the viscoelastic nuclear media and crowding. Such motion could also be attributed to diffusion near the glass transition (Doliwa and Heuer, 1998; Parry et al., 2014), or in disordered media (Havlin and Ben-Avraham, 2002). However, different proteins exhibit distinct characteristics of motion, which depend on their ability to interact with different nuclear elements (DNA, RNA) (Hansen et al., 2020). This suggests that transient interactions may be the principle determining nuclear mobility (Woringer and Darzacq, 2018).

An important feature of mammalian nuclei is that they contain membrane-less subcompartments such as the nucleolus, Cajal bodies, and nuclear speckles (Mao et al., 2011). These compartments can now be imaged with high resolution, and their shape and precise geometry can be measured (Cho et al., 2018). Those nuclear zones are characterized by an increased concentration of some components (proteins, RNA). Such an organization of the mammalian nucleus calls for focusing on geometrical models where sections of the nucleus are tiled with a high concentration of binding sites (Figure 7B). Indeed, it has been shown that the anisotropy of CTCF mobility (Hansen et al., 2020) and Pol II at viral replication sites (McSwiggen et al., 2019) can be explained using such geometrical models. The phenomenology of protein motion and organization naturally arises in such a model: (1) a protein that moves outside a zone would appear to perform Brownian motion (Figure 7C). (2) A protein moving inside a zone is retained for some time, resulting in transient trapping (Figure 7C). This would be the case when there is an energy barrier for the protein to cross the periphery. (3) When a protein has managed to escape a zone, it is likely to reattach and be trapped again, rather than transitioning to another nearby zone (Figure 7C) (Amitai, 2018). Reattachment and retention would both result in apparent subdiffusive, anisotropy motion but unlike fractional Brownian motion, it will not be scale free but depend on the size of the zone.

Recently, it has been proposed that parts of the nucleus are organized as phase-separated bodies or liquid droplets (Feric et al., 2016; Lin et al., 2015). How could one distinguish such an organization from other forms of protein clustering or aggregations? Geometric diffusion models may help us categorize these different modes of protein organization. Indeed, because of the phase boundary, one would expect the retention mechanism (Figure 7C) to be a dominant feature in the motion of proteins in liquid droplets. This can be assessed by measuring the diffusion coefficient within and without a zone (Peng and Weber, 2019), and characterizing other features of mobility such as anisotropy and the diffusion type. Conversely, in protein cluster/hub/aggregate, the reattachment mechanism (Figure 7C)

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could be the dominant determinant of the phenomenology of protein mobility. A future challenge would be to develop tools to invert the dynamics and distributions of protein, chromatin, and RNA, to estimate the geometry of such zones, and how they control various cellular processes.

Chromatin Contact Pattern Recognition in Hi-C: Insights from Protein Structure Prediction

In the last two decades, the introduction of chromatin conformation capture (3C) (Dekker et al., 2002) has lead to rapid development of techniques for the identification of 3D chromatin contacts at varying scales and genomic coverage (Dostie et al., 2006; Fullwood et al., 2009; Lieberman-Aiden et al., 2009; Mifsud et al., 2015; Zhao et al., 2006). Among these 3C techniques, Hi-C allows for the detection of whole-genome 3D chromatin contact maps, giving hints to large scale (kb-to-Mb) chromatin spatial organization and interaction (Figure 6C). These include the identification of global contact similarity (compartments A/B) and local contact insulation (TADs). This is analogous to the protein structure determination problem, where besides crosslinks, contact maps are also derived from nuclear magnetic resonance or covariance found in the alignment of homologous sequences. Unlike proteins, where there is often an experimentally determined structure, it is not clear how to validate these methods for chromatin. However, the analytical techniques and experiences from the field of protein folding might be applicable to elucidating and understanding chromatin structure.

One way to mine hidden meaningful patterns is to directly apply unsupervised methods like principal-component analysis (PCA) or clustering. This kind of approach has been used to classify compartments A and B in many cases (Fotuhi Siahpirani et al., 2016; Lieberman-Aiden et al., 2009), and subcompartments sometimes (Rao et al., 2014). However, these approaches do not necessarily correspond to biologically meaningful results. For example, the principle components that represent compartments A/B and those that represent chromosome arms might mix for human samples where the first two components have similar eigenvalues (Liu et al., 2018), challenging the compartment identification in these cases and highlighting the necessity of compartment segregation level evaluation. Moreover, these unsupervised contact pattern recognition methods in Hi-C focus mostly on the most dominant eigenvalues and their corresponding patterns, which give rich domain-level information. In the field of protein structure prediction, long-range contacts are often assumed to be sparse and emerge after removing the low rank dominant eigenvalues (Qin and Colwell, 2018; Zhang et al., 2016). Like protein, the sparse part in Hi-C matrices might contain key patterns and may contribute to direct contacts and therefore possibly lead to more accurate structure determination. Methods like robust-PCA (Candès et al., 2011) that decompose matrices into the low-rank and sparse components, inverse covariance, which essentially downweighs the largest eigenvectors, and balanced network deconvolution (BND) (Sun et al., 2015) are tools worth exploring for Hi-C contact pattern investigation and are promising for mining hidden information besides the relatively general domain-level patterns. The methods have been used to infer direct gene regulatory networks and interactions from gene co-expression data (Markowetz and Spang, 2007).

Another way is to observe the contact map directly and describe the contact patterns with parameters based on the observations. TADs are observed as contact blocks near the matrix diagonal and have strong intra-domain contacts and weak inter-domain contacts. Their identification is therefore based on block properties such as downstream-upstream difference near boundaries (Dixon et al., 2012), relative insulation from other regions (Crane et al., 2015; Van Bortle et al., 2014), and other matrix properties (Rao et al., 2014). These methods are dependent on contact map resolution and might give varying TAD sizes due to the hierarchical nature of TAD structures.

Similar to protein structure reconstruction, one-dimensional information like epigenetic and/or genomic information can assist identification of different contact patterns, further helping their discrimination. This one-dimensional information may help three-dimensional structure recognition in two ways: it can act as integrated marks or labels in itself or provide hints for uncovering new contact patterns. For example, association of regions with different DNA methylation level to chromatin contact map have been able to uncover different TAD organization patterns, which can be further identified by linear discriminant analysis (Xie et al., 2017) or neuron network classifier (Liu et al., 2018). Integrated epigenetic information have been shown to predict compartments and subcompartments (Di Pierro et al., 2017; Fortin and Hansen, 2015), and, at a smaller scale, TADs and interaction hubs (Huang et al., 2015), indicating their possible role as explicit structure labels in contact pattern recognition. Notably, chromatin structure is highly hierarchical, therefore, the importance of different epigenetic and sequential marks may vary in predicting structures at different scales. By labeling the contact map with one dimensional marks, the originally unsupervised contact pattern mining may switch to a well-aimed image (or network) classification problem.

Computational Imaging for Improved Throughput of DNA Damage Assays

DNA damage causes genomic instability and is a major driver of cancer and premature aging (Hoeijmakers, 2009). Damage can be induced from endogenous sources, such as reactive oxygen species, or environmental sources including UV and other radiation as well as some of the thousands of chemicals present in industrial processes. For example, diethylhexyl phthalate (DEHP), the most abundant plasticizer used in the production of polyvinyl-containing plastics, increases germline genome instability (Cuenca et al., 2020). As such, understanding how exposure to agents in our environment induces DNA damage is key to revealing the underlying basis for environment-promoted cancer. However, it is difficult to quantify DNA damage at low dose levels since the observed effects can be very small. While there are several approaches for quantifying DNA damage, by far the most sensitive is to assay for the frequency of damage using fluorescent microscopy. This is usually done using a marker for DNA damage, for example, fluorescently labeled DNA repair proteins such as 53BP1 that form DNA damage foci (Rothkamm et al., 2015).

To resolve foci in the nucleus, imaging needs to be done at very high-spatial resolution, preferably in 3D with large enough sample populations to build up robust statistics (Waddduwage et al., 2015). These competing demands translate to a

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trillion-pixel imaging experiment (for 10 million cells in 3D at diffraction-limited resolution), usually performed using wide-field microscopes (WFMs) equipped with megapixel-cameras. With an ~100 mW laser and a 10 ms exposure time, imaging takes about 3 hr. The most basic camera-based microscopes are WFMs followed by spinning-disk confocal microscopes (SDMs). WFMs are simple and power efficient but don't provide the depth selectivity needed for 3D imaging. SDMs use a micro-lens array to perform confocal imaging and have excellent depth resolution. SDMs are also power efficient due to the use of micro lenses (Pawley, 2010). More recently, light-sheet microscopy (LSM) (Ahrens et al., 2013) and structured illumination microscopy (SIM) (Schermelleh et al., 2008) have gained popularity for 3D imaging. While LSM suffers from insufficient depth resolution, SIM could be a competitor to SDM for DNA damage imaging (Choi et al., 2015; Wadduwage et al., 2015). Interestingly, certain variations of SIM satisfy the requirements for compressive sensing (Duarte et al., 2008; Wadduwage et al., 2019), which could, in theory, reduce the measurement time by almost an order of magnitude. But a demonstration for DNA damage imaging has been prevented by the challenges in image reconstruction of foci in the nucleus.

Compressive sensing powered SIM computationally reconstructs the image with the help of prior information about image features. Recent developments in machine learning, such as deep convolutional neural networks (dCNN), can potentially learn all image features in a 3D dataset (Gupta et al., 2019; Wei et al., 2019). While dCNNs are yet to be demonstrated for DNA damage imaging at subcellular resolution, traditional machine learning approaches have shown useful for DNA damage quantification at tissue level at low resolutions (Wadduwage et al., 2018). Thus, we anticipate that the combined power of deep CNNs with compressive SIM will change the imaging instrument landscape. Moreover, CNNs have started to replace almost all image processing algorithms in computer vision (Voulodimos et al., 2018) and will soon be commonplace in fluorescent imaging. Such a cascade of reconstruction and processing CNNs may also provide better compression, reducing the imaging time to just a couple of minutes. This sea change in image acquisition times will have a number of drastic effects on high throughput screening: (1) drug screens for DNA damage will be completed far more quickly, and (2) since acquisition time will no longer be a limiting factor, small effects of drugs or environmental conditions on DNA damage levels only detectable with large sample sets will be determined accurately. While this section discussed CNNs in the context of DNA damage, we note that this is only one application of this technology and that many biological imaging processes will be greatly facilitated by implementing compressive sensing or CNNs into their image analysis pipelines.

Moving from Cells to Organisms

The cutting-edge technologies transforming the chromatin field discussed above have been developed and deployed primarily in cell culture. These protocols are now translating to *in vivo* studies, and the impact this will have on organismal and developmental biology, regeneration, pathology, and evolution cannot be overstated (Figure 1). Functional genomic methods assessing transcriptional and epigenetic states such as RNA-seq, Hi-C, bisulfite sequencing, and ChIP sequencing (ChIP-seq) have been revolutionary in these fields. However, the large amount of tissue required and the heterogeneity of tissues has made parsing these data difficult and time consuming. Recent advances in single-cell technologies and *in vivo* imaging techniques change the experimental landscape by providing spatial resolution and sampling of rare subpopulations of cells, essential to understanding developmental and physiological states.

Drosophila has provided an amenable context for translating many of these new methods from cell culture to the organism (Chen et al., 2018a; Cusanovich et al., 2018; Lim et al., 2018). The extensive history of developmental genetics and the regularity of the early syncytial embryo supports this transition. Super-resolution microscopy methods have allowed for *in situ* imaging of enhancer-promoter interactions in combination with nascent transcript detection in fixed tissue (Mateo et al., 2019). In addition, live-imaging enhancer-promoter dynamics in the *Drosophila* embryo, in combination with cell-culture work, is overturning our understanding of long-distance chromosomal interactions (Heist et al., 2019; Lim et al., 2018).

Single-cell sequencing advances have already proven to refine cell- and stage-specific epigenomic states in the developing retina, cardiac tissue, neural crest, forebrain, and immune system (Jia et al., 2018; Norrie et al., 2019; Preissl et al., 2018; Yoshida et al., 2019). This enhanced resolution identified previously unknown cell types and transitory states in the process of differentiation. Greater clarity of cell trajectories is a powerful and essential step in the advancement of tissue engineering and regenerative biology (Kim et al., 2019; Scott et al., 2019). In addition, combinatorial epigenetic and transcriptional states will allow for more sensitive assessment of disease phenotypes, having immediate clinical implications for low-tissue pathological testing (Granja et al., 2019; Strzelecka et al., 2018).

These technologies also offer significant advances to the field of evolution and ecology. The limitation of genetic and epigenetic investigation across the phylogenetic tree has been the need for community investment to generate mutant and transgenic lines, specific antibodies, and sequencing resources. Long-read technology and chromosomal assembly methods have made genome assembly of non-traditional model organisms relatively common. New genomes in conjunction with new methods such as scRNA-seq and ATAC-seq have made cutting-edge transcriptional and epigenomic data accessible in the least experimentally accessible taxa in the tree of life, opening new fields of evolutionary and ecological inquiry (Gehrke et al., 2019; Lewis and Reed, 2019; Madgwick et al., 2019; Reynoso et al., 2019; Weizman and Levy, 2019). Furthermore, greater taxonomic sampling is revealing the evolution of genome organization and epigenetic mechanisms and how these manifest and contribute to organismal plasticity and complexity (Lu et al., 2019; Marlétaz et al., 2018).

Conclusions

This review combines snapshots of technologies that will be crucial for the advancement of chromatin biology combined with perspectives on key research areas where these techniques

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can be applied such as organismal and developmental biology or phase separation. We hope that this effort will prime the creativity of the next generation of chromatin biologists and drive deeper collaborations between scientific communities.

ACKNOWLEDGMENTS

This work arose from discussions during meetings of the "Boston Chromatin Club" and was supported by the Office of the Provost, Faculty of Arts & Sciences, and Center for Advanced Imaging at Harvard University. A.S.H. acknowledges support by the National Institutes of Health (NIH) NIGMS Award no. R00GM130896. J.D.B. and A.S.L. acknowledge support by the Allen Distinguished Investigator Program through the Paul G. Allen Frontiers Group and support from the NIH New Innovator Award (DP2). A.C. acknowledges support from MBL Physiology Course 2019 during which she had many fruitful conversations with Clifford P. Brangwynne, in whose group she worked on using optical tweezers to measure nuclear mechanical properties. T.N. is supported by JSPS Overseas Research Fellowships and a National Institutes of Health grant (NIH GM044794). We are grateful to Nancy Kleckner for helpful discussions and financial support to L.C. Studies described above include unpublished work by the author (L.C.) and by other current and former members of the Kleckner laboratory: Zhangyi Liang, Nadine Vincenten, Akwasi Andrews Agbleke, Maria Mukhina, and Frederick Chang and are supported by a grant from the NIH (GM 025326). A.D.S. is supported by Pathway to Independence Award NIGMS (K99GM123195). K.M.K. acknowledges support from the Office of the NIH Director 1DP5OD023111-01 and Harvard University.

AUTHOR CONTRIBUTIONS

Conceptualization, A.S.; Writing – Original Draft: A.S., A.A., A.S.H., T.N., H.A.S., and J.-H.S.; A.S.H., J.-H.S., and L.C. wrote the "Chromatin imaging" section; A.C. and A.D.S. wrote the "Tools to manipulate chromatin" section; D.W., S.L., and S.O. wrote the "Image processing and data analysis" section; J.D.B., A.S.L., and K.M.K. wrote the "Tools to study chromatin at the single-cell level" section; Writing – Review and Editing, all authors; Table, A.A.A., Overview Figure, A.A.A., A.S., and K.M.K.; Funding Acquisition, A.S; Proofreading, all authors.

DECLARATION OF INTERESTS

J.D.B. holds patents related to ATAC-seq. The remaining authors declare no competing interests.

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Please cite this article in press as: Agbleke et al., Advances in Chromatin and Chromosome Research: Perspectives from Multiple Fields, Molecular Cell (2020), https://doi.org/10.1016/j.molcel.2020.07.003

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Please cite this article in press as: Agbleke et al., Advances in Chromatin and Chromosome Research: Perspectives from Multiple Fields, Molecular Cell (2020), https://doi.org/10.1016/j.molcel.2020.07.003

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