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A guide for single-particle chromatin tracking in live cell nuclei.

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Short running title: single-particle live cell chromatin tracking

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Abbreviations:

A small protein tag based on human O6-alkylguanine-DNA-alkyltransferase (CLIP-tag)

Clustered regularly interspaced short palindromic repeats (CRISPRs)

Double-strand break (DSB);

Electron Multiplying charge-coupled device (EMCCD)

Fluorescence in situ hybridization (FISH);

Förster resonance energy transfer point accumulation for imaging in nanoscale topography (FRET-PAINT)

Highly inclined and laminated optical (HILO)

Linear assignment problem (LAP)

Light-emitting diode (LED);

Lattice light sheet (LLS)

Multitemporal association tracking (MAT);

Multiple-hypothesis tracking (MHT);

Mean square displacement (MSD)

Region of interest (ROI)

Photo-active fluorescent protein (PAFP)

Point accumulation for imaging in nanoscale topography (PAINT)

Probabilistic data association (PDA)

Point spread function (PSF)

Quantitative Point accumulation for imaging in nanoscale topography (qPAINT)

Randomly cross-linked (RCL)

RNA polymerase II (RNAPII)

Scientific Complementary metal-oxide-semiconductor (sCMOS)

Signal-to-Noise Ratio (SNR)

Single Particle tracking (SPT)

Transcription factor (TF)

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Trimethoprim chemical tag (TMP)
Total internal reflection fluorescence microscopy (TIRF)

ABSTRACT.

The emergence of labeling strategies and live cell imaging methods enables the imaging of chromatin in living cells at single digit nanometer resolution as well as milliseconds temporal resolution. These technical breakthroughs revolutionize our understanding of chromatin structure, dynamics and functions. Single molecule tracking algorithms are usually preferred to quantify the movement of these intranucleus elements to interpret the spatiotemporal evolution of the chromatin. In this review, we will first summarize the fluorescent labeling strategy of chromatin in live cells which will be followed by a systematic comparison of live cell imaging instrumentation. With the proper microscope, we will discuss the image analysis pipelines to extract the biophysical properties of the chromatin. Finally, we expect to give practical suggestions to broad biologists on how to select methods and link to the model properly according to different investigation purposes.

Keywords: chromatin motion, DNA damage, particle tracking, single molecule imaging.

1. Introduction.

The chromatin is a complex and dynamic structure packed by eukaryotic DNA with histones. Genomic functions including gene expression, DNA synthesis, and DNA repair, are regulated by the higher order chromatin organization. The chromatin structure and architecture is strictly controlled and constantly remodeled as cells differentiate, divide, and respond to genomic insults (Auerbach et al., 2009, Chien and van Noort, 2009, Clapier and Cairns, 2009, Misteli, 2007, Salvador Moreno et al., 2019, Vidi et al., 2014). While DNA linearly encodes genetic information, offering a template for the production of RNAs and proteins, the temporal and spatial organization of chromatin plays a fundamental but critical role in determining intranucleus activities and gene stability (Cuvier and Fierz, 2017, Dion and Gasser, 2013). On the most basic level, fast (microseconds to seconds) local structural fluctuations in nucleosomes transiently expose buried DNA sites, thus providing temporary access to interaction sites (Choy and Lee, 2012). Similarly, chromatin fibers are subject to rapid conformational dynamics (Kruithof et al., 2009, Li et al., 2016, Poirier et al., 2009). This intrinsic motion of the chromatin directly affects molecular interactions at the local level by dictating the accessibility of the DNA for various epigenetic effectors, chromatin regulators, and transcription factors (TFs). Therefore, the nanoscale spatiotemporal profile of chromatin may modulate the interaction of DNA with regulatory molecules, thus impacting the global patterns of gene expression (Bintu et al., 2018, Boettiger et al., 2016, Grant et al., 2018, Xu et al., 2018).

Chromatin domains typically display stochastic motions (Marshall et al., 1997, Weber et al., 2012, Wiesmeijer et al., 2008), with rare exceptions of directional movements (Levi et al., 2005). The kinetics of chromatin is best described by sub-diffusive (or anomalous) diffusion models (Fierz and Poirier, 2019, Shukron et al., 2019), with fiber movements constrained by (1) the polymeric nature of the molecule, (2) finite dimensions of the cell

nucleus, (3) physical obstacles within the nucleus, (4) viscosity of the nucleoplasm, and (5) intra- and inter-fiber interactions. However, the field is cluttered with apparently contradictory findings, partly due to the scarcity of suitable methods and physical models to study chromatin dynamics.

Most of what we know about local chromatin motion in chromatin remodeling was derived from ensemble measurements, which provide a population-averaged picture of biochemical access to the chromatin. Recent advances using single molecule approaches have enabled the direct observation of dynamics of individual molecules and allowed measurement of their respective spatiotemporal localizations and provided functional clues/implications. Particularly, time-course recording of the movement of chromatin loci provides quantitative measures which allow us to assess the spatiotemporal dynamics of the chromatin and deduce the biophysical properties of the chromatin. We expect to review the latest progress of measuring the chromatin motion in live cells.

2. Intranucleus imaging

2.1 Labeling strategies of chromatin

Combined with some advanced microscopy systems and single molecule tracking algorithms, an effective labeling technique is required to illuminate chromatin structure and dynamics successfully. In general, visualizing chromatin is achieved by tagging a fluorescent reporter onto the principal actors of central dogma in eukaryotic cells: DNA, RNA and protein. Below, we primarily review various classical and emerging chromatin labeling strategies based on proteins and DNA (summarized in Table 1).

2.1.1 Chromatin associated protein-based labeling strategies

As the constitutive basic unit of chromatin, core histones (H2A, H2B, H3 and H4) that are tightly wrapped by DNA molecules are the common labeling targets for imaging. In fixed cells, chromatin associated protein can be visualized through immunostaining with antibodies (Conic et al., 2018, Ricci et al., 2015, Xu, Ma, 2018). In live cells, histones can be directly fused with fluorescent protein (FPs) (Belmont, 2001, Das et al., 2003, Kanda et al., 1998, Mora-Bermudez and Ellenberg, 2007), while this method has the limit of controlling labeling density, therefore it is not quantitative when labeling the highly expressed proteins. Photo-active fluorescent protein (PAFPs) were developed to address this limitation meanwhile allowing real-time characterization of protein clustering quantitatively. This method can be combined with other technical strategies, such as single molecule tracking and super resolution imaging (Cisse et al., 2013, Manley et al., 2008, Nozaki et al., 2017). For example, the Zhuang lab engineered mMaple2, mMaple3 which exhibited substantially reduced dimerization tendencies (Wang et al., 2014) - live cell imaging time is prolonged to 5 hours without detectable phototoxicity or photobleaching by adding precise imaging system settings etc. (Baker et al., 2010). An alternative histone labeling approach for live cells is to use prevalent self-label tags (Liss et al., 2015, Stagge et al., 2013), including Halo Tag, Snap Tag, CLIP tag, and TMP Tag. With the advantages of small size, brightness, photostability, monomerization, and adjustable fluorescent dye concentration, they have shown superior performance in single-molecule imaging (Grimm et al., 2017, Nagashima et al., 2019, Nozaki, Imai, 2017).

Based on the same principles, other genomic elements are fluorescently labeled to assess structure and dynamics including telomeres(Avogaro et al., 2018), centromeres(Avogaro, Querido, 2018, Gasser, 2002), H-NS or HU in *E. coli* (Wang et al., 2011), heterochromatin protein(Hu et al., 2013), and transcription factors (TFs) (Elf et al., 2007, Gebhardt et al., 2013). Despite the stability and efficacy of protein-based labeling methods, it suffers from several major drawbacks. For example, these methods are only suitable for global or non-specific investigation and overexpressed FPs are likely to induce labeling artifacts.

2.1.2 DNA-based labeling strategies

Non-sequence specific: A number of cell-permeable DNA dyes, such as Hoechst (Green and Sambrook, 2017), 4',6-diamidino-2-phenylindole (DAPI), YOYO-1 and DRAQ5, are available to strongly bind to DNA offering a simple labeling method with great photostability in fixed cells. Similar to the principle of DNA binding dyes, dNTPs which formed DNA backbone can also be fluorescently labeled to demonstrate chromatin structure(Bu et al., 2019). However, one notable disadvantage of these fluorescent DNA-dyes is that they provide low or no specificity, which is of essential for further elucidating how the structure of DNA affects its functional outcome.

Sequence specific: The development of Fluorescence in situ hybridization (FISH) allows one to detect and locate sequence-specific DNA/RNA in fixed cells by utilizing probes with a sequence complementary to the target sequence (Bayani and Squire, 2004, Bellevue et al., 2015). In the past few years, FISH has been extensively optimized: Mer-FISH, QFISH, smFISH, seqFISH, CASFISH strategies improved the specificity, sensitivity, multiplexing, throughput and qualitative analysis (Deng et al., 2015, Haimovich and Gerst, 2019, Iourov, 2017, Moffitt and Zhuang, 2016, Shah et al., 2018, Xia et al., 2019, Zhang et al., 2018). Although it has advanced the field significantly, FISH suffers from inability to elucidate dynamics in the living environment.

Therefore, locus-specific labeling in live cells is in high demand. For decades, it has been readily achieved by either inserting artificial DNA sequences next to target gene intrusively, such as the repressor–operator array system: Lac operator (LacO) and Tet operator (TetO) system, or modified genome-editing tools with inactive nucleases such as zinc finger proteins (ZFPs), transcription activator-like effectors (TALEs), clustered regularly interspaced short palindromic repeats (CRISPRs). The lacO-LacI-FP and tetO-TetI-FP systems are derived from the lactose operon and the tetracycline operon of *E. coli* respectively. Lac and Tet repressor proteins are fused to FPs as the tracking foci and recognize the repressor tandem repeat sequences previously inserted next to the position of interest (Ding and Hiraoka, 2017, Loiodice et al., 2014). Notably, multiple systems and repressor segments are allowed in a single cell simultaneously to enhance system multiplicity and fluorescent amplification (Backlund et al., 2014, Roukos et al., 2013, Tasan et al., 2018). In addition, point accumulation for imaging in nanoscale topography (PAINT) techniques are attractive for single molecule localization microscopy because of it was unrestricted by the photon budget. First demonstrated by Jungmann et al. in 2010 (Jungmann, Avendano et al. 2016), DNA based PAINT was explored and improved over the past decade. Quantitative PAINT (qPAINT), Förster resonance energy transfer PAINT (FRET-PAINT) (Jungmann, Avendano et al. 2016), and Exchange PAINT have been de-

veloped to generalize the use of the DNA origami to reveal the cellular interactions, while the off-target challenges are still existing (Nieves, Gaus et al. 2018). Furthermore, the ParB-INT system can similarly insert ~ 1 kb INT element, a small dose to get a strong signal without interfering with chromatin dynamics and transcription (Saad et al., 2014).

Nevertheless, any intrusive DNA insertion can potentially perturb function and cause alterations to chromatin locus position and mobility. The adoption of non-intrusive ZFPs, TALEs, and CRISPR-dCas9 circumvented the constraints, without any artificial DNA insertion (Chen et al., 2016, Lindhout et al., 2007, Ma et al., 2013). These systems rely on a series of modular proteins with specific DNA recognition, where the endonuclease-deficient proteins usually were fused with FP as the detectable signal. Among these labeling strategies, CRISPR imaging systems are receiving increased attention - Chen et al. re-engineered the type II system to visualize both repetitive elements in telomeres and non-repetitive MUC4 (Chen et al., 2013). To further achieve multicolor imaging in the CRISPR system, one strategy is to use fluorescent Cas9 orthologs from different bacteria species simultaneously, such as *Streptococcus pyogenes* (SpCas9), *Neisseria meningitidis* (NmCas9) and *Streptococcus thermophilus* (St1Cas9) (Ma et al., 2015); another alternative strategy is engineering sgRNA into a scaffold RNA (scRNA) which involves encoding information of the gene of interest and multiple fluorescent reporters (Zalatan et al., 2015). Numerous recent modifications based on CRISPR imaging systems, for example, CRISPR-display (Shechner et al., 2015), CRISPRainbow (Ma et al., 2016), suggesting promising applications in investigating chromatin organization and visualizing genome instability and rearrangement (Chen, Guan, 2016).

2.1.3 mRNA-based labeling strategies

Multiple optical techniques have been implemented to study the subcellular localization of mRNA to provide insights into the transfer system in the cell (Tutucci et al., 2018). As mentioned above, single-molecule fluorescence in situ hybridization (smFISH) can be used to localize individual mRNAs in fixed samples. The fundamental information of gene expression in bacteria, yeast, or mammalian cells has been explored experimentally by various techniques based on smFISH. While the transcription, splicing and packaging of the mature mRNA are dynamic processes. The engineered fluorescent RNA aptamer system derived from bacteriophage has been inserted along with target mRNA to the untranslated region and encoded genetically, which are used to tag mRNA in the living system, including MS2-MCP system, PP7-PCP system, λ N, Bgl stem-loops, λ boxB RNA, and U1A tagging (Tutucci, Livingston, 2018, Tyagi, 2009). The kinetics of mRNP-NPC interactions and translocation was demonstrated by combining FISH and MS2 at the three-dimensional (3D) single mRNP level (Mor, Suliman, 2010). Although the RNA aptamer system has been the standard strategy to imaging mRNA in live-cell for a long time, endogenous mRNA imaging in the live system has not been achieved. Even CRISPR systems are facing the potential interference to chromatin when being used to image endogenous RNA. Zhuang etc. developed an RNA-aptamer-based two-color CRISPR labeling system to enhance the signal-to-background by attaching the MS2 or PP7 aptamers to different locations on the sgRNA, and further experimental tests were needed to explain the full potential of the new system (Wang et al., 2016).

2.1.4 Nanobody labeling strategies.

In many preclinical studies of regenerative medicine, quantum dots (QDs) imaging technology has been widely used, primarily due to the good chemical and photo-stability, high quantum yield and size-tunable light emission (Han et al., 2019, Matea et al., 2017, Zavoiura et al., 2020). Based on QDs, various systems have attempted to trace RNA in live cell. One type of QD, nanobeacon, was reported by Ma etc. to track the uncoating process of single HIV-1 via precisely conjugating the black hole quencher (BHQ1) and phosphorothioate comodified DNA onto CdTe:Zn²⁺ QDs (Ma et al., 2019). Stem cells are labeled *in vitro* and *in vivo* by various groups of QDs, such as CdSe/ZnS QDs, AgInS₂/ZnS QDs, graphene QDs, Ag₂S QDs, AgInTe₂QDs (Yukawa and Baba, 2018). With the development of novel organic QDs, the potential of QDs for clinical applications have been strongly supported. Nanobodies are becoming a type of promising toolkit for the diagnosis and therapy of diseases nowadays with the unique advantage of small size and quick clearance from blood. Various studies have developed specific nanobodies for molecular imaging (Bao et al., 2021), Moeglin etc. presented a novel nanobody which precisely visualizes phosphorylated histone H2AX by TIRF which enables the analysis the dynamics of particular chromatin modification in individual cancer cells in various formats of DNA damage assays (Mor et al., 2010).

2.2 Instrumentation for intranucleus imaging.

Among many factors that determine the accuracy of chromatin loci movement, spatial resolution is the parameter that dominates the reliability of downstream biophysical analysis (Burov et al., 2013). Therefore, for most reported work of intranucleus chromatin imaging, objectives with high numerical aperture (N.A.>1.2) are employed. Meanwhile, as indicated in the last section, multiple fluorophores were employed in chromosome labeling strategies to ensure adequate fluorescent signal; therefore, there are few limitations on imaging modalities, and various microscopes can be used for chromatin imaging. For many intranucleus imaging experiments, an epi-illumination fluorescent microscope, which is equipped with a modern camera (EMCCD, or sCMOS) (Fig. 1A), can provide time-course recording of live cells. In addition, the emerging LED light sources are replacing laser excitation in these microscopes, which significantly reduces the cost of the microscope (Albeanu et al., 2008, Hattori et al., 2009, Zheng and Qian, 2013). Another widely applicable imaging system that can be used for chromatin imaging is the confocal microscope (Fig. 1B), which provides good z sectioning capability that can reduce the out-of-focus background. It should be noted that the applicability of confocal microscopy for chromatin motion is due to the relative slow motion in the nucleus ($D < 0.001 \mu\text{m}^2/\text{s}$) (Shukron, Seeber et al. 2019). However, intrinsic limitations of epi-illumination and confocal microscopes, such as photo-bleaching/photo-toxicity and poor temporal resolution, restrict their applications for sensitive chromatin imaging at the single molecule level. The usual alternative to epi-illumination microscope, the total internal reflection fluorescence microscopy (TIRF) (Fig. 1C), was capable of capturing photons from a single fluorescent molecule; but it is not suitable for chromatin imaging in nucleus because of the poor penetration depth (~200 nm).

The emerging light sheet illumination offers proper solutions to the above-mentioned challenges and achieves the balance among spatiotemporal resolution, photo-bleaching effect, and background deduction (Fig. 1E). In 2008, a simple but efficient light sheet illumination method was proposed to generate a highly inclined and laminated optical

sheet (HILO) for single molecule imaging (Tokunaga et al., 2008). As the high spatial resolution can be retained thanks to the high N.A. objective, the HILO illumination significantly reduces the total illumination doses on the cell but at the cost of largely reduced field of view (Fig. 1D). Since then, the light sheet illumination draws tremendous attention due to its advantages in reducing the phototoxicity, enhancing the sectioning capability, and finally the potential for live cell three-dimensional (3D) imaging. Early development of the light sheet microscope, which used two orthogonal objectives (N.A. ~ 0.65) for excitation and/or emission, has demonstrated great performance for 3D imaging of tissues, embryo, and organs (Keller et al., 2015, Keller et al., 2010, Keller et al., 2008, Tomer et al., 2011). However, its application in single cell imaging and even single molecule imaging was prevented by the geometric hindrance when using two high N.A. objectives. Recent efforts have been focusing on the development of new light sheet modalities for single cell 3D imaging. An AFM cantilever was initially used to reflect the illumination light sheet by 90 degrees to bypass the geometric hindrance (Gebhardt, Suter, 2013). A similar idea was also proposed by microfabricating a reflecting chip next to the sample reservoir (Galland et al., 2015). Both approaches can generate a thin sheet of light with a beam waist of $\sim 1\mu\text{m}$. Furthermore, the lattice light sheet (LLS) microscope, which generates the light sheet with Bessel beam, significantly reduces the thickness of the light sheet to 300 nm (Chen et al., 2014). The LLS has demonstrated the best performance at various features, such as the spatial resolution, temporal resolution, phototoxicity and sensitivity (Gao et al., 2019). However, the generation of optical lattices requires an optical train that is complex, expensive and lossy. A very recent work proposed a simple and universal optical process, which was described by a mathematical theorem as field synthesis, to create any type of light sheet, including the lattice light sheet (Chang et al., 2019). In principle, this approach can be integrated with any light sheet imaging system to enable the lattice light sheet imaging.

The further development of imaging modalities for chromatin imaging will aim at a light sheet imaging that can balance various imaging parameters and can provide a time-course recording of chromatin motion in 3D. Generally, the chromatin can be modeled as beads and string. To determine polymer models that infer the actual behavior of chromatin inside the nucleus, one must be able to compare extracted physical parameters from real experimental chromatin data with those predicted from the model. However, the physical model fails to infer the actual chromatin dynamics primarily due to the inconsistency of modeling and experiments. This happens because the 2D imaging data cannot offer the positional information of chromatin motion in 3D, while the physical modeling is in 3D. A recent study by the Misteli group directly compared 2D and 3D distance measurements in the cell nucleus (Finn et al., 2017). Although 2D measurements (i.e. positions or trajectories projected in a single plane) are practical and generally well-suited for flat nuclei from monolayer cell cultures, 3D measurements are needed to improve accuracy. This is particularly true for round nuclei (as for 3D cultures) where the relationship between 2D vs 3D distances deteriorates, and for short distances ($< 5\mu\text{m}$) where the average 2D/3D discrepancy is $\sim 30\%$ (Finn et al., 2017). Moreover, it is important to consider nuclear inhomogeneity in all 3 dimensions. For motion measurements, the 2D/3D discrepancy of $\sim 30\%$ might not be very significant, when considering the local fluctuation, $\sim 50\text{nm}$, the 3D nature cannot be ignored.

It should be noted that the imaging instrumentation and analysis methods discussed here share many similarities with the localization-based super resolution microscopes, such as (Fluorescence) Photoactivation Localization Microscopy (PALM or fPALM), and Stochastic Optical Reconstruction Microscopy (STORM). Therefore, the advancement in the super resolution imaging would also benefit the intranuclear chromatin imaging. One typical example is the application of aspheric optics. Originally, this approach was utilized by the super resolution imaging to convert the z-axis position of the single molecule to the distorted point spread function (PSF) on the x-y plane, through a cylindrical lens (Huang, Wang et al. 2008). This method was then further developed in single molecular imaging/tracking to register the 3D position of a moving molecule through a rotational PSF (Greengard, Schechner et al. 2006, Pavani, Thompson et al. 2009, Badieirostami, Lew et al. 2010, Thompson, Lew et al. 2010). In addition, the utilization of super resolution imaging on the live cell single molecule imaging would produce high density trajectories of the molecule, this enables the integration of biophysical analysis, such as a stochastic model of nonequilibrium motions, to recover the forces, subcellular organizations, the diffusion kinetics, and many other biophysical features at an unprecedented spatio-temporal resolution (Hoze, Nair et al. 2012, Holcman, Hoze et al. 2015, Hoze and Holcman 2015). It is anticipated that the recent progress in quantitative super resolution imaging, such as qPAINT (Jungmann, Avendano et al. 2016) (Culley, Albrecht et al. 2018, Mockl, Pedram et al. 2019), will be the next booster engine for the live-cell chromatin imaging.

3. Single molecule tracking.

3.1 Nucleus/cell registration.

It is notable that cells are constantly moving with live cell imaging techniques. This raises big challenges for image analysis and data extractions, since the intracellular movements and dynamics could be distorted or erased by the global movement of the cell body. For example, chromatin inside the nucleus move and vibrate at a very low speed. Therefore, correcting the movement of a live cell properly is critical for obtaining accurate its interior components mobility data (Fig. 2A). The general approach, named cell registration, is particularly critical for intranucleus motion since the cellular motion is usually comparable or quicker.

In general, image registration can be classified as rigid registration and non-rigid (deformable) registration. Rigid registration is relatively simpler since it only deals with simple uniform transformation, while non-rigid registration has to identify the deformation between the images and match their features or boundaries accordingly. A registration algorithm can usually be composed of three main components: (1) Identify similarities, such as features and geometric shapes, between a given image and its reference images; (2) Create a deformation field, or a transformation model. (3) Optimize the deformation field to maximize the similarity (Crum et al., 2004).

Various methods have been developed to extract the similarities from several images, The commonly used methods include: (1) Feature matching; (2) Segmentation based registration; (3) Geometric transformation; (4) Contour/Boundary tracking. Feature match-

ing techniques identify certain features inside the images, which is usually realized by matching the similar features of their intensity profiles (Kim et al., 2011, Yang et al., 2008) or Fourier transformed images (Maintz and Viergever, 1998, Zitová and Flusser, 2003). This method is efficient, however it may interfere with the movements of the cell content and not able to preserve the difference of the interior of the cell.

Segmentation is a relatively time-efficient and well-developed method for current image registration. Segmentation helps by segmenting the image into small segments, usually by signal-based algorithms or model-based algorithms. Signal-based segmentation focuses solely on the signal information provided by the image, while having no knowledge on any shape or structure of the segments, which is ideal for non-rigid registration. On the other hand, model-based segmentation algorithms apply shapes or active contour, it is mostly effective when the image does not experience a major deformation, which means it is mostly suitable for rigid registration. After segmentation of the image, the registration algorithm can then find the similarities between segments in different image, and create the deformation field between the images (Maintz and Viergever, 1998).

Geometric transformation, namely applying translation, rotation or affine, into the image such that it can be more similar to the reference one. This method is mostly useful in rigid registration because of the low complexity nature in rigid transformation (Zitová and Flusser, 2003). This method can also be implemented in deformed registration with the help certain techniques, by examining the cell as an elastic body, both linear and nonlinear, or model it as a viscous fluid flow, one can estimate the transformation of a certain cell within a temporal difference and creates the deformation field (Sotiras et al., 2013).

The contour/boundary tracking method mainly focuses on the exterior of the cell, and tries to identify the change of the cell boundary between images. Considering that most cells do not have highly deformed exterior, the algorithm mainly fits the cell using active contour or snakes, which helps find the boundary of the cell, and then obtain the deformation field which minimizes the large deformation of the boundaries of the cell image and matches them to the reference cell image. This is highly useful when researchers want to preserve the interior features of the cell, since it only focus on the exterior deformation of the cell (Maintz and Viergever, 1998, Sotiras, Davatzikos, 2013).

Current cell registration algorithms mostly consist of a combination of the methods mentioned above according to different situations. Besides, some research also explores the possibility of integrating machine learning and artificial neural networks into the registration methods, for instance learning to obtain the deformation field, which can largely improve the efficiency and accuracy of cell registration.

3.2 Principle of the single molecule tracking.

3.2.1 Single molecule localization

For single particle tracking, localization is a critical step to extract the particle coordinate with subpixel accuracy. Since any error in this step would significantly affect all later steps, localization is one of the main sources of error for single molecule tracking analysis. Extra care and fine parameter tuning are usually required. Typical single molecule

localization composes of three steps: apply filters, identify ROI (region of interest), and estimate coordinates (Fig. 2B).

Noise presents in any kind of experimental data, independent of the imaging strategy used. Noise could originate from the sample, such as background fluorescence and fluorescence instability of the label; it also could be generated by the camera, such as shot noise and pixelation noise. Applying filters to the raw data ensures proper noise deduction and signal enhancement. Background reduction is often achieved via the application of median, wavelet-based (Hua et al 1997), moving average i.e., boxcar (Smith 2011), and rolling-ball filters (Sternberg 1983). Noise reduction can be achieved using a variety of techniques, from simple convolution with a Gaussian kernel for white noise reduction (Smith 2011) to more sophisticated methods which utilize neural networks (Kefer 2020). The signal stands out from the background after applying one filter or a combination of several filters. Once filters are applied the ROI needs to be identified from the whole image. Frequently used methods include local maxima finding, and thresholding. Parameters need to be carefully tuned to ensure reliable ROI identification. Improper parameter setting can lead to blinking behavior of the ROI, ultimately leading to improper quantification. The final step is to extract subpixel accuracy center coordinates for each ROI. Point spread function (PSF) fitting is generally used for this purpose, especially for individual particles that have sizes smaller than the diffraction limit, since they behave as point source emitters and generate blurred images described by the PSF of the microscope (typically Gaussian function). However, when the particle size is getting larger and less symmetric, other methods also apply, such as intensity-based centroid calculation or simply taking the coordinates of the local maxima (Chenouard 2014).

Single molecule localization could be done simply in one pass or iteratively. The iterative localization is preferred for the case of high particle density (Serge et al., 2008), where the low intensity particles could be easily obscured by the nearby high intensity particles. The strategy of the iterative localization is that it would first locate high intensity particles, subtract their corresponding PSF from the image, then make low intensity particles prominent and detectable. The process would be repeated until all the particles are detected, and only noise remains. Detecting all particles exhaustively improves statistics extracted during single particle tracking.

3.2.2 Building trajectory and calculation.

After applying a detection method on time-lapse 2D digital images, we are often tasked with reconstructing particle trajectories from the sampled coordinates (Fig. 2C). Associating detected particles can be difficult – the procedure is complicated by high particle density, low SNR, and motion blur. Indeed, high particle density can introduce ambiguity into the tracking procedure and therefore erroneous tracks. Limits in lateral and/or axial resolution can result in apparent particle merging which often cannot be distinguished computationally, even in principle. Depending on the tracking algorithm, incorrect localization of particles due to low SNR can affect track quality directly or indirectly. Furthermore, tracking errors can result from temporary or permanent particle disappearance which can be due to photo-bleaching, blinking, or motion along the axial dimension. These complications may result in large temporal gaps or early truncation of an otherwise

continuous trajectory. Tracking algorithms which are robust to the mentioned complications are highly desirable, as they allow observation of the interactions between particles and collection of large volumes of data across a wide variety of experimental setups. However, results of a recent particle tracking challenge suggested that there is no method that is universally superior across all experimental scenarios (Chenouard 2014). Rather, the applicability of several methods should be assessed according to the specific experimental context. This makes the problem of developing a universal tracking method even more difficult given that the accuracy of tracking is inherently dependent on experimental parameters. Nevertheless, we can assess commonly used algorithms to reduce the number of possible approaches for the case of chromatin tracking in the nucleus.

(1) Probabilistic Methods

A diverse set of particle tracking algorithms utilize probabilistic models of particle motion in order to add detected particles to existing tracks. Perhaps the most fundamental particle tracking method in this category is the nearest neighbor (NN) linking algorithm first introduced by Crocker and Grier. The algorithm constructs particle trajectories by assuming that the ensemble consists of non-interacting indistinguishable particles undergoing Brownian motion. As a result, the displacement of each particle follows a Gaussian distribution, parameterized by the diffusion coefficient and the time-resolution of the sequence. The most probable assignment of detected particles to existing tracks can then be found by maximizing the product of several Gaussian distributions (Crocker and Grier, 1996). An attractive feature of this algorithm is that it is relatively simple to implement; however, assumptions that underlie the method impose limitations. In particular, if a typical displacement δ in one time step is comparable to the typical inter-particle spacing, tracking becomes highly error prone (Crocker and Grier, 1996). In other words, when particles are simultaneously densely distributed and undergoing fast diffusion the algorithm can fail to build accurate trajectories due to the ambiguity introduced by overlapping trajectories. This also applies to the limit of low frame rates when small displacements are not recorded by the sensor. Importantly, the method alone cannot handle cases where particles disappear permanently, temporarily disappear due to blinking, photo-bleaching, or missed localization (Sbalzarini and Koumoutsakos, 2005).

More modern probabilistic approaches address spatial and temporal uncertainties in particle localization by formulating tracking within a Bayesian framework. Such methods handle these uncertainties introduced by measurement noise by defining a posterior distribution on the variables e.g., coordinates, given measurements extracted from image data (Godinez and Rohr, 2015). For example, the Kalman filter has been used to estimate true particle trajectories from measured trajectories under the assumption of Gaussian white noise. Interestingly, in a recent particle tracking challenge this method has performed quite well for accurate track reconstruction, particularly in regimes of low SNR (Chenouard 2014). Other solutions to the particle tracking problem search for tracks which are globally optimal. These algorithms search for the optimum set of tracks within the set of all possible tracks, considering the length of the entire movie. This approach is adopted by so-called multi-hypothesis tracking (MHT), which performs multi-dimensional assignment by delaying association decisions until all data association ambiguities are resolved (Reid 1979). However, a significant drawback is that the method is

very computationally expensive - the number of track hypotheses grows exponentially with increasing particle number (Kim 2015). Nevertheless, the method can still be used and, similar to probabilistic data association techniques, has scored quite well in reconstructing tracks at low SNR (Chenouard 2014).

(2) Combinatorial Optimization

From the perspective of computational complexity, the particle tracking problem is a NP-hard problem with factorial complexity for three or more frames (Shafique and Shah, 2005). The complexity of the general problem therefore requires that we can only find suitable approximations. An alternative path towards solution uses combinatorial optimization for particle tracking – a class of methods which find optimal tracks by minimizing a specially chosen cost-function. More generally, combinatorial optimization techniques can be distinguished based upon two primary criteria: the cost function and the optimization procedure. Optimization can be carried out between adjacent frames (greedy two-frame assignment) or across a window of frames (multidimensional assignment). Bipartite graphs have been used to solve two-frame assignment (Chertkov, Kroc, 2010, Sbalzarini and Koumoutsakos, 2005) while directed k-partite hypergraphs to solve multi-frame correspondence (Shafique and Shah, 2005). The cost-function could for example be a function of distance between detection instances. This is the case when the particle tracking problem is framed as a linear assignment problem (LAP) where the cost-function is defined as a linear combination of imaged-based measurements.

An example solution for particle tracking which uses the LAP formalism is the feature point algorithm developed by Sbalzarini and colleagues. Their algorithm uses a cost function based upon quadratic differences in position and intensity moments (Sbalzarini and Koumoutsakos, 2005). In general, the LAP uses a cost matrix analogous to the association matrix used in bipartite graphing methods. However, the matrix is divided into four quadrants: one containing the costs to link with the next frame, not to link with the next frame, not to link with the previous frame, and one auxiliary block required by the LAP formalism (Winter, Fang, 2012). In a more recent implementation of this method, greedy two-frame assignment is followed by linking together track segments produced by the first solution. This subsequent assignment then results in temporally global optimization and a closer approximation to MHT (Jaqaman 2008). There is little doubt that this approach improves upon algorithms relying solely on greedy assignment strategies such as NN-linking and provides a tractable approximation to MHT, allowing the robust tracking of particles under high-density conditions (Jaqaman 2008). However, performance of these algorithms appears to suffer in the limit of low SNR compared to probabilistic methods (Chenouard 2014).

(3) Use of open-source tools for chromatin tracking

The relatively slow ($D < 0.001 \mu\text{m}^2/\text{s}$) and sub-diffusive behavior chromatin in the nucleus has important implications for the choice of a tracking algorithm. Obviously, it should also be noted that the quality of predicted tracks is limited by the performance of the chosen detection algorithm. In this section, metrics of performance are based on tracking alone and may change if the detection method is sub-optimal. A number of open-source

software tools exist for particle tracking which can greatly expedite data processing. Here, focus will be placed on tools which can be readily integrated with existing image processing programs and libraries such as ImageJ, MATLAB, and Python. In the limit of slow diffusion, we expect that SNR as well as particle density will be the primary contributors to tracking errors. When the SNR is unity, Kalman-filtering and probabilistic data association have been shown to perform quite well in predicting ground-truth tracks over a wide range of particle densities (Chenouard 2014). However, for increasing SNR, other algorithms surfaced such as MHT, feature-point, and the Viterbi algorithm (see Chenouard 2014 Fig. 2b). At any rate, the performance margins in reproducing ground-truth tracks were sometimes narrow. Moreover, Jaqaman and colleagues developed uTrack – a particle tracking toolkit written in MATLAB which finds a solution to the particle tracking problem using a form of combinatorial optimization (Jaqaman 2008). More specifically, the authors implementation performs two-frame linking by solving a sequence of linear assignment problems which solve two-frame assignment followed by closing of temporal gaps and handling merging/splitting. This package is well-standardized and may be a good choice for MATLAB users; however, optimal reconstruction of ground-truth tracks is restricted to the limit of high SNR under Brownian motion (Chenouard 2014).

Other frameworks have been developed as ImageJ plug-ins which can provide an efficiency boost when other image processing and detection algorithms precede the tracking process. A prime example is the TrackMate plug-in which is highly flexible between different use-cases as it provides users with multiple choices of linking algorithms: (i) linear assignment (ii) Kalman filter (iii) NN-algorithm (Tinevez 2017). The first is derived from the uTrack algorithm developed by Jaqaman and colleagues and will therefore apply for high SNR over a wide range of particle densities. The second is a probabilistic association method and thus could be a good choice in the limit of low SNR. The third is reserved for more fundamental tracking scenarios where a simplified algorithm will suffice. Furthermore, the Spot Tracking tool and ParticleTracker are ImageJ plug-ins which can be used for particle tracking. The former relies on the MHT tracking algorithm, which has been shown to perform quite well for low SNR when particle density is also low (Chenouard 2014). The latter uses the feature point tracking algorithm developed by Sbalzarini and colleagues which could be a strong choice when SNR is relatively high over a wide range of particle densities. Finally, for Python users, the TrackPy library developed by Allan and colleagues provides tracking functionality based on the NN algorithm developed by Crocker and Grier. This tool can be particularly useful for Python users with existing familiarity with the scientific Python stack. The greediness of this algorithm may restrict its use to ideal cases when particle motion is Brownian and particle density is low. However, its performance across various experimental scenarios has yet to be fully analyzed.

4. Biophysical analysis and chromatin modeling.

4.1 Physical parameters extracted from the SPT analysis.

Based on trajectory data, quantitative information involved in the motion of the particle can be extracted (Fig. 2D). Be mindful that the chromatin structure is very complicated

and cannot be represented by the diffusion of individual particles exactly. However, by treating each fluorescent foci as a point mapped in space and time, we can come to an understanding whether the motion is constrained (sub-diffusion), super diffusion or follows normal diffusion. Therefore, by only using position (via particle tracking algorithms) and frame rate we are able to uncover the following parameters.

The most common method to analyze single particle trajectories is to calculate the mean square displacement (MSD) which represents the scaling of the average squared displacement from the origin as a function of time

$$MSD = \frac{1}{N} \sum_{t=0}^{N-1} (r(t + \tau) - r(t))^2 \quad (1)$$

The sampling time given by τ is interpreted as the framerate of the camera. Certain studies have shown that the motion observed is different for certain values of τ (Amitai et al., 2017, Shukron and Holcman, 2017a). Once the MSD curve is established, effective diffusion coefficient D and Anomalous Exponent α values are obtained by curve fitting techniques using:

$$D(\tau) \propto \tau^{-\alpha} \quad (2)$$

where m is the number of dimensions, which reflects the velocity of diffusion and the degree of confinement of individual particles respectively. Importantly, according to different parameter values, further interpretation and classification of different diffusion modes of particles can be achieved (Wasim and Treanor, 2018, Zhong and Wang, 2020). By comparison of the D value of all particles within nucleus, the spatial heterogeneity can be clarified. Meanwhile, different α values can be fitted to different diffusion models, trajectories with $\alpha = 1$, the motion is thought to be Brownian motion, when $\alpha > 1$, it said to be directed motion, $\alpha < 1$ is classified as confined (or immobilized) diffusion. While the calculation of D and α particularly reliant on the length of trajectory via the suitable number of data points, recent investigations built the diffusion color maps of fluorescently histones by computing vast amounts of super resolution trajectories to reveal some mechanism or interaction in cell biology (Amitai, Seiber, 2017, Barth et al., 2020, Nozaki, Imai, 2017). Moreover, unlike the case of particles driven by free diffusion, which can simply be described by diffusion constant D , confined motion needs more parameters to describe. Therefore, the proposal of radius of confinement (R_{conf}) allows measuring the constraint of particles, while the limitation of R_{conf} calculation is that it relies on the plateau of the MSD curve (Lerner et al., 2020, Wieser and Schutz, 2008). Alternative similar plateau-independent parameter called the length of constraint (L_c) was introduced in some studies as well by

$$\sqrt{\frac{1}{N_p} \sum_{i=1}^{N_p} (R_i - \langle R \rangle)^2} \quad (3)$$

where N_p is the number of data points in the trajectory (Amitai et al., 2015, Verdaasdonk et al., 2013). Via comparing with the radius of the nucleus, both of R_{conf} and L_c provide the estimation of the radius of constraint or volume of space explored by the locus over time. However, in most of intranucleus biological models, the dynamic of molecule was

affected by many factors, not only crowding constraints also some tethers or other fibers, the well-known one is chromatin polymer model. To estimate resulting restriction from these tethers, the effective spring coefficient K_c which can give measurement of the averaged external forces affecting the interesting locus can be observed by

$$K_c \approx \frac{1}{2(N_p-1)} \sum_{i=1}^2 \sum_{h=1}^{N_p-1} \frac{R_c^i((h+1)\Delta t) - R_c^i(h\Delta t)}{D_c \Delta t} \quad (4)$$

K_c has been widely used combined with the predicted polymer model to unveil the effect of local tethering interaction in nuclear (Amitai, Toulouze, 2015, Hauer et al., 2017, Shukron, Seeber, 2019, Verdaasdonk, Vasquez, 2013).

4.2 How to link the experimental data with the chromatin modeling.

It is well-known that DNA double helix structure consists of millions of base pairs chained together by the sugar-phosphate backbone. At the same time, DNA is a molecule built by many similar monomers bonded together and it is therefore a natural choice to analyze DNA with a polymer model. The simple polymer model of chromatin starts with the Rouse polymer model which discretized it into monomers connected by springs, similar to the string-of-beads type structure that has been observed under the electron microscope. Various forces acting on chromatin result in constrained dynamics, so the anomalous exponent for the dynamics of a chromatin locus has a large variability. To further modeling refined chromatin properties according exponent α , the β -polymer model was described which is a construction with a prescribed exponent α . Mid-range and long-range interactions between monomers can be captured by it, not just those between the closest neighbors as in the Rouse polymer model, α shows a decay curve with the distance along the chain in this case. $\alpha=0.5$ in the traditional Rouse polymer model, while the β -polymer model can be selected to further discuss for the cases that α is in the range of 0-0.5 (Amitai and Holcman, 2013, Amitai, Seeber, 2017, Hajjoul et al., 2013). Thus, local interaction between monomers can be recovered from the anomalous diffusion exponent by the construction. Furthermore, as the polymer model usually folds at different spatial scales and generates various sizes of loops, a simulation model that adds connectors between randomly chosen non-nearest neighbor monomer pairs called randomly cross-linked (RCL) polymer models was present. α is reduced lower than 0.5 by adding connectors in this model, meanwhile recent evidence suggests that the number and distribution of connectors affects physical parameters in many ways, but it is still under explored domain (Shukron and Holcman, 2017a, b). One notable thing is that the RCL polymer model might be an essential model to study the dynamics in the process of CTCF and cohesion regulating chromatin loop stability (Hansen et al., 2017). In addition, the complicated case that subdiffusion with an exponent $\alpha > 0.5$ can also appear in some polymer models, different types of forces are possible involved, such as deterministic force or directed motion or other monomer interactions (self-avoiding and bending interactions).

Based on linking chromatin predicted β -polymer model parameters and experimental data (D , L_c , α , K_c), chromatin expansion and its behaviors at double-strand breaks has been studied by improved time-lapse imaging regimens (Amitai, Seeber, 2017). To conclude,

the proposal of chromatin polymer models accompanied with parameters extracted from particle trajectories in recent works is moving forward to provide mechanistic insights, meanwhile allow prediction and reconstruct chromatin dynamic and organization, and main driving forces responsible for nuclear organization and function.

5. Biological Applications

Chromatin motion and DNA damage. The subject that has been studied and made great progress through single molecule tracking is DNA damage and repair (Cheblal et al., 2020, Hauer, Seeber, 2017). Multiple labs have proved that chromatin mobility increased dramatically after double-strand breaks (DSB), it also affects the efficiency of DNA repair which is homologous recombination (HR) (Adam et al., 2016, Chiolo et al., 2011). This hypothesis has been supported by the case that tracking related proteins fused with fluorescence protein (Dion et al., Hauer, Seeber, 2017). Another studies monitor trajectories of LacI-GFP protein bound to an array of lac operators (lacO) that is inserted near the cut site at a high temporal resolution using improved time-lapse imaging regimens, they predict chromatin domain expansion near a DSB and damage extrusion from the domain (Amitai, Seeber, 2017) (Fig. 3A).

Chromatin motion and DNA replication. The chromatin motion in G1-phase shows higher degree than S in both mammals and yeasts, which suggests DNA replication contributes to reduction of chromatin movement (Walter et al., 2003, Wiesmeijer, Krouwels, 2008). A study proved that nucleolar integrity constraint the mobility of these foci by monitoring LacO-tagged locus before and after Scc1-TEV subunit of cohesion in S and G phase (Dion et al., 2013) (Fig. 3B). The dynamic in deferent cell cycles also suggest that It is chromatin organization and dynamics play a critical role in gene transcription.

Chromatin motion and transcription. The conventional mechanism of chromatin motion in chromatin remodeling is that the local short-range motion of the chromatin leads to the transient exposure of nucleosome DNA through partial unwrapping of the DNA, such transient structural change will determine the accessibility and interactions of TFs, polymerases and other chromosomal effectors (Fierz, 2016, Fierz and Muir, 2012). However, recent findings using single molecule imaging have revealed that the chromatin motion is entangled with the binding affinity and association/disassociation rates of various effectors, such as TFs and other DNA binding proteins (Gu et al., 2018, Hihara et al., 2012, Nagashima, Hibino, 2019). It is reported that the mobility of the chromatin and associated elements increase concurrently with transcriptional activation (Nagashima, Hibino, 2019). Furthermore, it is found that active RNA polymerase II (RNAPII) globally constrains chromatin movements, and RNAPII inhibition or its rapid depletion released the chromatin constraints and increased chromatin dynamics (Nagashima, Hibino, 2019) (Fig. 3C).

6. Conclusions and Perspectives.

In summary, this paper provided the technical guidance towards the live-cell imaging and associated biophysical analysis of chromatin motion. A systematic introduction of the intranuclear labeling methods discuss the available molecular strategies that enable the

imaging of chromatin with high sensitivity and high specificity. Proper imaging systems were also discussed based. A technical guidance of live-cell image processing and single-particle tracking analysis was provided to offer practical and reliable choices of the analysis pipeline and proper parameters. The physical properties extracted from these single particle trajectories provide independent and complementary information towards the chromatin architecture, dynamics, and function. Taken together, these technologies are further elucidating the interaction between chromatin dynamic and chromatin organization, diverse cellular functions, biological processes.

The fast advancement in super resolution imaging and digital image processing/analysis definitely will elevate our understanding towards the chromatin architecture and chromatin dynamics. The recent emerging super resolution imaging techniques would enable the dynamic visualization of the chromatin structure at a video rate(Li, Tingey et al. 2021, Wu, Han et al. 2021), this will help to integrate the chromatin motion with the conformational change of the chromatin (Lakadamyali and Cosma 2020). In addition, the utilization of artificial intelligence in image processing has revolutionized the digital image processing and analysis. The application of convolutional neural network (CNN) has been highly used to remove the noise of any fluorescent images(Weigert, Schmidt et al. 2018). Recently this approach has been used in the single molecule tracking, and suggested the great potential in restore the chromatin motion at a weak excitation (Kefer, Iqbal et al. 2021). This indicates that the local chromatin motion can be evaluated at a short time scale (~milliseconds).

Although the synergistic development in advanced labeling strategies and imaging techniques enable direct visualization of chromatin structure and dynamics from the scale of nucleosomes to the entire nucleus, it remains unclear how to reconcile the fine chromatin structures with the dynamic nature. A possible solution towards this challenge is to integrate super-resolved chromatin organizations with the chromatin motion(Xu, Ma et al. 2020) (Xu, Ma et al. 2018). By PALM/STORM imaging of H2B fused PAGFP or Halo Tag, fast and slow nucleosome movement are categorized and revealed aspects of organization of heterogeneous chromatin domains for better understanding genome organization (Nozaki, Imai, 2017). There are studies in yeast and mammal also showed centromeric/ yeast SPB and telomeric tethering constrained chromatin movement significantly (Bronshtein et al., 2015). The intermolecular tethering of heterochromatic loci to larger nuclear structures enhances constraint as well, such as the nuclear periphery or nucleoli (Chubb et al., 2002). In addition, chromatin modeling can facilitate the interpretation of the chromatin motion at the local level and in the long range. More importantly, the chromatin motion data could be integrated with the chromatin conformation data, which is captured through HiC, to develop a unified model of the chromatin (Huang, Li et al. 2020, Woodworth, Ng et al. 2021). Such a model would take into account the chromatin motion with the chromatin conformational change, and will allow for the study of casual relationships between chromatin motion and other subcellular activities, such as epigenetic modification, transcription, DNA replication, DNA damage and repair.

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DATA AVAILABILITY STATEMENT.

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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TABLE TITLES & FIGURE LEGENDS.

Table 1 Summary of different chromatin labeling methods.

	Method		Specific		Other Advantages	Limitation	Reference
	Immunostaining		No	Intrusive	Easy to implement	Only allow to study global structure, bulk labeling, fixed	(Choi et al., 2011, Ricci Manzo, 2015)
Protein based	fluorescently tagged histone	Histone-FP/PAF P	No	Intrusive	Stable fluorescence, any cell type, live cell, signal amplification	Limited number available	(Baker, Buckheit, 2010, Das, Payer, 2003, Kanda, Sullivan, 1998, Mora-Bermudez and Ellenberg, 2007, Sparks et al., 2018, Wang, Moffitt,

						2014)
	Histone-self labeling Tag	No	Intrusive	Microbial enzymes, controllable density, any cell type, live cell	Limited number available	(Grimm, Muthusamy, 2017, Nagashima, Hibino, 2019, Nozaki, Imai, 2017)
	Nanobody	Yes	Intrusive	small size, excellent solubility, superior stability	Artificial locus	(Bao, Tang, 2021, Mor, Suliman, 2010)
	DNA binding Dye	No	Intrusive	Easy to implement	Bulk labeling, some only for fixed cell, potential interfere chromatin	(Lukinavicius et al., 2015, Martin et al., 2005, Schoen et al., 2011)
	FISH	Yes	Intrusive	Stable fluorescence, great multiplexing capacity, numbers of modification	Fixed sample	(Beliveau, Boettiger, 2015, Deng, Shi, 2015, Haimovich and Gerst, 2019, Iourov, 2017, Moffitt and Zhuang, 2016, Shah, Takei, 2018, Xia, Fan, 2019, Zhang, Cerqueira, 2018)
DNA based	Repressor-operator array systems	LacO-LacI-FP	Yes	Intrusive	Great brightness, live cell	Artificial locus, with DNA arrays insertion, potential inter-
						(Ding and Hiraoka, 2017, Liodice, Dubarry,

						ferre chromatin	2014)
		TetO-Tetl-FP	Yes	Intrusive			(Loiodice, Dubarry, 2014, Tasan, Sustackova, 2018)
Based on genome editing tools	ZFP	Yes	Non-intrusive	Stable fluorescence, live cell, without any insertion, numbers of modification, good multiplexing capacity	Restricted to repetitive sequences, hard to implement, time-consuming		(Lindhout, Fransz, 2007, Lindhout et al., 2010)
	TALE	Yes	Non-intrusive				(Ma, Reyes-Gutierrez, 2013, Thanisch et al., 2014)
	CRISPR-dCas9	Yes	Non-intrusive		Restricted to PAM sequences, local DNA unwinding/triple helix formation, potential background noise		(Anton et al., 2014, Chen, Gilbert, 2013, Deng, Shi, 2015, Knight et al., 2015, Ma, Tu, 2016, Shechner, Hacisulleyman, 2015)
RNA based	Based on FISH						
	smFISH Mer-FISH QFISH seqFISH CASFIH	Yes	Intrusive	Stable fluorescence, great multiplexing capacity, numbers of modification	Time-consuming to design probes, fixed sample		(Beliveau, Boettiger, 2015, Deng, Shi, 2015, Haimovich and Gerst, 2019, Iourov, 2017, Moffitt and Zhuang, 2016, Shah, Takei, 2018, Xia, Fan, 2019, Zhang, Cer-

Fluorescence aptamer system	MS2	Yes	Non-intrusive	Stable fluorescence, live cell numbers of modification,	Potential interfere chromatin, not for endogenous RNA	veira, 2018) (Laprade et al., 2020, Wang, Su, 2016)
	PP7					
Based on CRISPR system	spCas9	Yes	Non-intrusive	Stable fluorescence, live cell, without any insertion, numbers of modification, good multiplexing capacity	Huge molecular weight will increase the burden of the labeled mRNA and influence the dynamics of the mRNA	(Nelles et al., 2016, O'Connell, 2019, Schindele et al., 2018)
	c2c2 (RNase Cas13)	No	Non-intrusive	Excellent fluorescence properties, good chemical and photostability, high quantum yield and size-tunable light emission	Cytotoxicity, heavy metals	(Han, Zhao, 2019, Ma, Mao, 2019, Yukawa and Baba, 2018)
Quantum dots						

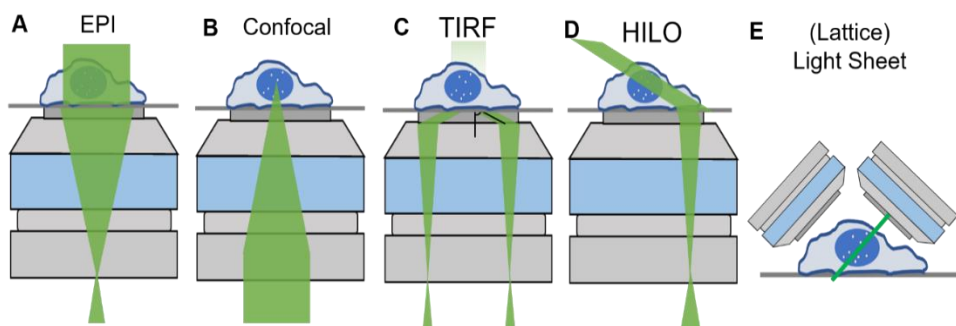


Figure1. Schematic drawing of different imaging modalities for intranuclear imaging. (A) epi-illumination and wide-field image acquisition; (B) Scanning the focused lattice

ser beam in a confocal microscope yields great sectioning capability; (C) The schematic of total internal reflection fluorescence microscopy (TIRF); (D) The highly incline optical illumination offers the balance of sensitivity and photostability; (E) A light sheet microscopy with two orthogonal objectives as the best option for 3D intranuclear imaging; the lattice light sheet can be generated by sweeping the focused Bessel beam.

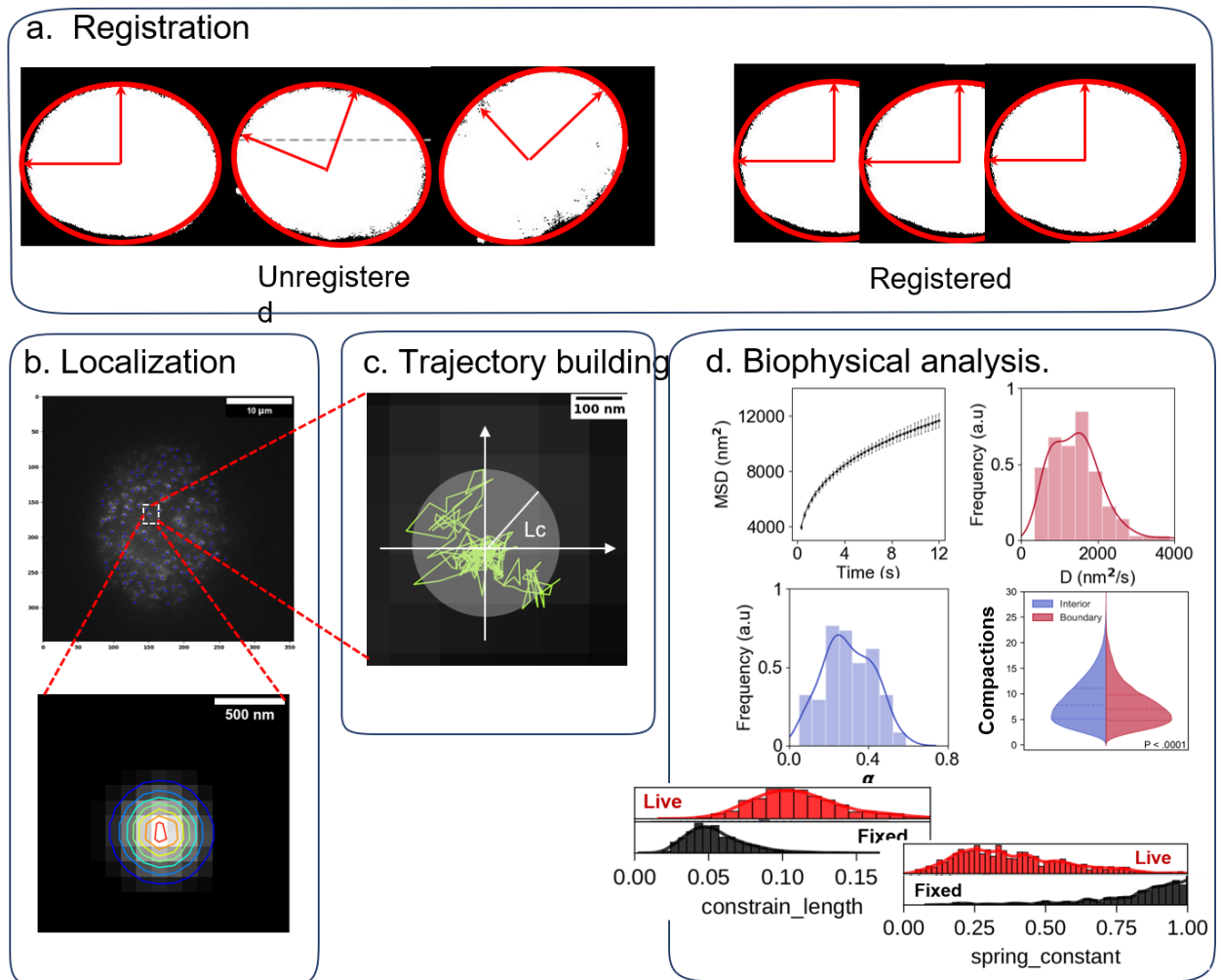


Figure 2. The workflow of the single particle tracking to analyze the biophysical properties of the chromatin motion. (A) Image registration is required to correct the motion of the nucleus in a time-course recording of the chromatin motion. (B) localization of individual chromatin loci is identified by the single particle detection algorithm. (C) the trajectory of a chromatin locus is reconstructed, and (D) associated biophysical analysis quantify the chromatin motion, including the diffusion coefficient, degree of the constraint, compaction, and stiffness.

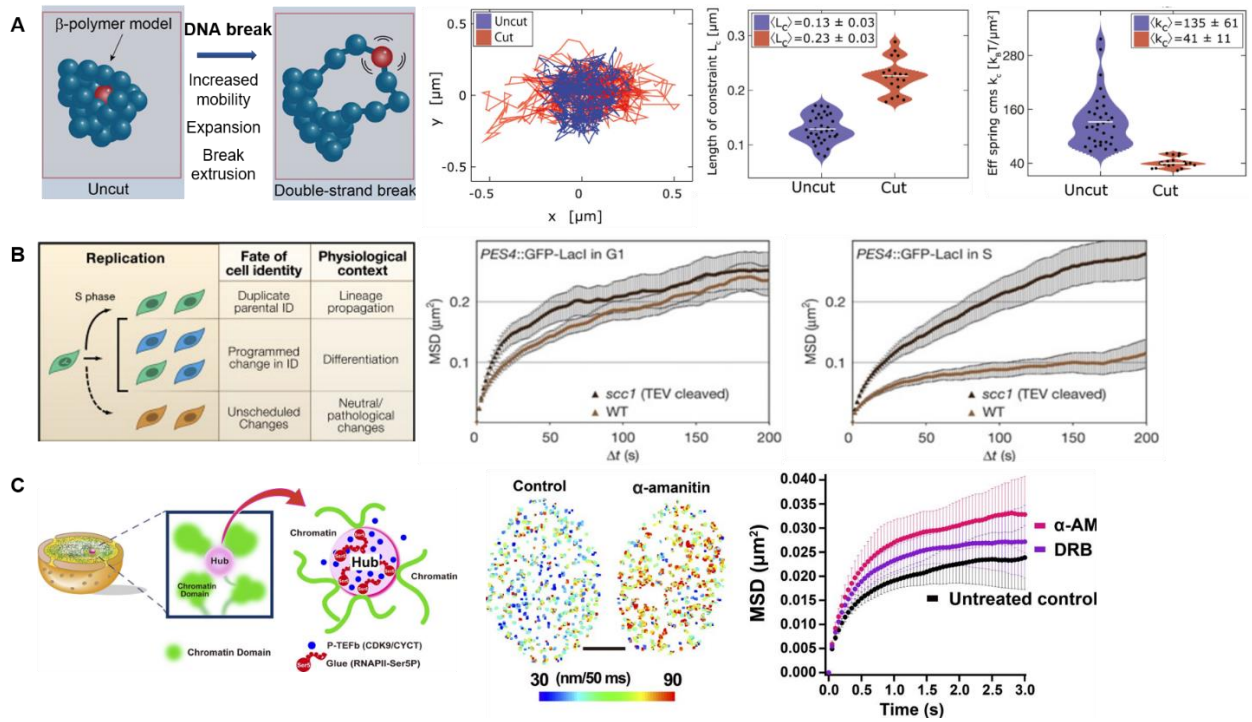


Figure 3. Intranucleus imaging reveals the correlation of chromatin motion with intracellular activities, such as DNA damage (A), DNA Replication (B), and Transcription (C).

(A) Left: the schematics of the DNA break leads to increased chromatin motion. Right: the comparison of L_c for MAT, derived from trajectories taken at $\Delta t = 80 \text{ ms} \pm 25 \mu\text{M}$ LatA before and after 2hr double strand break induction; all figures are from (Amitai, Seeber, 2017). (B) Different MSD curves of LacO-tagged PES4 in GA1461 (wild type) and after cleavage of Scc1 in G1 and S phase respectively; conceptual figure (left) is from (Groth, Rocha, 2007); two MSD figures (right) are from (Dion, Kalck, 2013). (C) Left: the chromatin heat maps of the nuclei treated with and without RNAPII inhibitors (α -amanitin); right: MSD plots (\pm SD among cells) of single nucleosomes in RPE-1 cells treated with RNAPII inhibitor (α -AM, DRB) or without inhibitors (control). All figures are from (Nagashima, Hibino, 2019)