

Super Resolution imaging of chromatin in pluripotency, differentiation, and reprogramming

Short Title: Identifying the nanoscale assembly of the chromatin fiber

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Abstract

Chromatin fiber organization in embryonic/adult stem cells and in somatic cells undergoing reprogramming to pluripotency is one important determinant of gene function. Until now the diffraction limit of light has limited the inspection of the chromatin fiber organization to a level sufficient to understand how it impacts gene function. The development of advanced microscopy methods, such as single molecule localization microscopy, has largely opened a new field of research providing us with the tools to visualize and quantitatively analyze chromatin fiber organization and thus gene activity at nanoscale resolution in single cells.

Introduction

Euchromatin and heterochromatin has been traditionally considered the active and repressive portion of the genome, respectively [1]. Spatial distribution of euchromatin or “open chromatin” and heterochromatin or “closed chromatin” in the nuclei has also been correlated with gene activity and function [1]. Although this categorization of the different portions of the genome is widely used, this is still an ambiguous definition and we need to use quantitative imaging methods to clearly identify at nanoscale resolution how the open/active and closed/silent chromatin state is organized.

A number of studies recognized that the chromatin state in embryonic and adult stem cells as well as in somatic cells reprogrammed to pluripotency is largely open and enriched in euchromatin [2]. However, many questions to dissect how the chromatin fiber changes in cells undergoing differentiation and/or reprogramming need to be answered:

1. Will the nanoscale visualization of the chromatin fibers provide us with novel knowledge on gene function?
2. Will the spatial position of gene loci be more/less informative than the local nanoscale organization of the fibers with regards to gene activity?
3. Will the nucleosome and transcription factor movements be essential to study gene function in the same way as epigenetics has been?

In this review, while focusing on stem cell differentiation/somatic cell reprogramming, we will summarize how the use of a number of advanced microscopy methods (Box 1) has at least partially addressed these open questions. We will draw some conclusions and further speculations on the future results that can be reached in this field, which is still in its infancy.

The chromatin fiber assembly: from the textbook view toward a novel model.

The chromatin fiber is formed by the DNA wrapped around the nucleosome octamers composed of H2A, H2B, H3 and H4 core histones [3] with the linker histone H1 compacting the chromatin by its binding to DNA entry/exit points of nucleosomes [4]. Using X-ray diffraction, the 10nm diameter DNA double helix was seen compacted into a higher ordered fiber of 30nm with a solenoid or two-start helix spatial arrangement [5, 6]. Cryo-electron microscopy, small-angle X-ray scattering imaging and computational modeling have instead revealed that the 10nm fiber can occasionally assemble into more condensed areas [7-9] and not in a 30nm solenoid fiber [10, 11].

Electron spectroscopic imaging (ESI) and three dimensional structural illumination microscopy (3D-SIM) provides us with information on the spatial organization of the chromatin fibers in the nuclei of stem and somatic cells.

Spatial organization, i.e. whether a gene locus is located at the periphery or in the center of the nucleus will influence its transcriptional activity [12]. Moreover HiC approaches have shown that specific inter- and intra-chromosome interactions can control gene expression [13]. Therefore, how the chromatin is spatially organized in embryonic stem cells (ESCs) and somatic cells has been a matter of deep investigation.

By ESI the fine nuclear organization of mouse ESCs and differentiated cells has been studied (Figure 1). In ESCs the chromatin is uniformly dispersed and composed of 10nm fibers, while in differentiated cells, such as mouse embryonic fibroblasts (MEFs) and lymphocytes, there is a large fraction of densely packed heterochromatin organized in domains or chromocenters or located in the nuclear periphery. Interestingly, in differentiated cells, part of the nuclear volume is largely devoid of DNA, while the DNA occupies large portions of the ESC volume [14].

By combining ESI with tomography, the structure of MEF heterochromatin regions that are enriched of H3K9me3 and of H4K20me3, were seen as highly compacted, however remarkably, these regions included only 10nm fibers and not 30nm fibers. MEFs-derived induced pluripotent stem cells

(iPSCs) did not contain compact heterochromatin domains and instead showed H3K9me3 and H4K20me3 enriched regions composed of dispersed 10nm fibers [14].

Furthermore, combining correlative light microscopy with ESI (LM/ESI), the structure of chromocentres in partially reprogrammed iPSCs was investigated and seen to pass from a very compacted state, resembling that of MEFs, to a state with loosely packed fibers in ESCs and fully reprogrammed iPSCs [15]. Interestingly, while the existence of dispersed chromatin in ESCs was already demonstrated by ESI before [16], using LM/ESI, Fussner and co-authors confirmed their observations made previously in iPSCs, i.e. that not only the 10nm fibers were present in densely packed chromocenters of partially reprogrammed iPSCs, but also that the heterochromatin enriched in H3K9me3 is compatible with dispersed chromatin and is composed of 10nm fibers in ESCs and fully reprogrammed iPSCs.

Overall during iPSC generation, a transition from highly compacted and folded 10nm fibers organized in chromocenters into a dispersed distribution of 10nm fibers has been postulated. Epigenetic modifications were hypothesized to be responsible for this transition.

The use of ESI has been important also to correlate the heterochromatin organization in mESCs with a novel function of Nanog, an essential stem cell factor. The chromatin in Nanog^{-/-} ESCs appeared less uniformly distributed and compacted at the nuclear envelope and within discrete chromocenters including high levels of H3K9me3. Compared to wild type ESCs, chromocenter number in Nanog^{-/-} ESCs was also considerably higher. Overall, Nanog was shown to be essential for the compaction and redistribution of the heterochromatin and therefore to maintain an open heterochromatin organization in ESCs [17].

The presence of 10nm-dispersed chromatin has been also found by analyzing the changes in the chromatin structure from one-cell stage to early postimplantation stage mouse embryos using ESI [18]. Quite expected, chromatin organization in embryo epiblast cells and ESCs was seen as indistinguishable, confirming that dispersed chromatin is a hallmark for pluripotency and undifferentiated stage.

Another important aspect of nuclear organization has been studied during pre-implantation bovine development. Using 3D-SIM the Nuclear Pore Complex (NPC) and the lamina were found to be homogenously distributed at all stages, while, intriguingly, NPCs were not homogenously distributed in the nuclear envelope of pronuclei and of 2- to 8-cell embryos [19]. Interestingly, envelope regions lacking the NPCs were also characterized by lack of DNA contacting the envelope. One can therefore speculate that this presence/absence of DNA at the envelope generates a diversity of gene activity across the different developmental stages.

The chromatin organization of ESCs versus that of adult stem and progenitor cells, such as HSCs, MPP, GMP, B and GM cells was in parallel investigated using high-resolution electron microscopy (EM) and soft X-ray tomography [20]. In this study it was shown that the amount of euchromatin correlated with the transition from the ESC state to the lineage committed state (passing from HSCs to mature myeloid and B cells). Moreover, the spatial

distribution of the heterochromatin correlated with differentiation state, with the heterochromatin layer increased at the nuclear envelope along with cellular differentiation. GMP cells were seen to deviate from this classification, since they did not accumulate much heterochromatin at their periphery. This might well be related with their high reprogramming potential [21].

3D-SIM and Transmission Electron Microscopy (TEM) also allowed assembling a topological chromatin density map based on DAPI intensity (Figure 1 and 2) to categorize the chromatin of human hematopoietic progenitors, myeloid precursors, monocytes and granulocytes in seven different compartments [22]. These compartments span from DNA free to decondensed chromatin to compacted chromatin clusters. Despite the differences in chromatin organization at the global level in the different nuclear types, markers of active chromatin, such as H3K4me3, RNA Polymerase II Ser2P and splicing speckles such as SC35, were imaged and mapped to be in the lower chromatin categories in all cell types. The silent chromatin mark H3K9me3 showed broader variability in its distribution but was enriched in the higher chromatin classes in all cell types.

The use of ESI, 3D-SIM and TEM largely open up the identification of the chromatin structure conformation in somatic cells, ESCs, iPSCs, early developmental embryos and lineage committed progenitors. In these studies reprogramming and pluripotency grade has been largely correlated with a dispersed distribution of the 10 nm chromatin fiber and with a not homogenous distribution of the NPCs in the nuclear envelope. Overall, these investigations suggested that the transition from somatic to pluripotent state or from embryonic to lineage-committed state are also due to the variation of the chromatin distribution and organization within the nuclei. One state or the other can be maintained by epigenetic modifications and transcription factors, such as Nanog.

The nanoscale structure of the chromatin fiber dissected through single molecule localization microscopy teaches about gene function

Using Stochastic Optical Reconstruction Microscopy (STORM), we recently investigated the chromatin organization in pluripotent and differentiated cells at nanoscale resolution [23]. STORM imaging allowed us to visualize with high molecular specificity that nucleosomes are arranged into discrete groups, which we called nucleosome clutches, in analogy to eggs clutches (Figure 1 and 2).

We quantified single molecule localization of the histone protein H2B in a variety of cells. Although we observed that in each single nucleus there is a large distribution of clutch sizes, we found that the median number of nucleosomes per clutch is cell state specific and strongly correlates with the pluripotency grade of each cell type.

Ground state mouse ESCs display nucleosome clutches containing few nucleosomes, which are loosely packed inside the clutches. This feature allowed us to identify a subpopulation of ESCs cultured in serum+LIF medium, which expressed reduced level of Nanog and that lost their ground state of pluripotency. Likewise, our quantitative analysis correlated with the results of

pluripotency tests and led us to discriminate high-grade pluripotent human iPSCs from low-grade clones [23].

We also studied the chromatin organization in somatic cells. Human fibroblasts (hFbs) and Neural Precursor Cells (NPCs) show bigger and denser clutches with respect to hiPSCs and mESCs, respectively. However after trichostatin A (TSA) treatment, which increases the amount of open and actively transcribed chromatin, the clutches in hFbs became smaller, less packed and more uniformly distributed within the nucleus. Furthermore TSA-treated hFbs showed a significant reduction of clutch density in the heterochromatin regions at the nuclear periphery. Polymerase II was more closely associated to the smaller clutches, suggesting that small clutches constitute the so typically called 'open and active chromatin'. On the contrary the heterochromatin regions in centromeres were enriched in larger and denser clutches, which thus form 'closed' chromatin [23].

Overall, our data identified a novel chromatin organization where the fibers are irregularly assembled in a specific somatic or pluripotent cell-associated state. Importantly, our studies defined quantitative measurements of what constitutes 'open' and 'close' chromatin in terms of nucleosome organization.

3D-STORM was also recently applied to image chromatin regions with different epigenetic states [24]. Using Oligopaint to label long genomic areas, different chromatin regions were classified as 'active' when the genomic loci were enriched for H3K4me2 or H3K79me3; as 'repressed' when enriched for H3K27me3 or Polycomb Group (PcG) proteins and as 'inactive' when presenting a predominance of unmodified histones and a depletion of PcG proteins. Despite a cell-to-cell and a significant locus-specific variation, each epigenetic state was found to correspond to a distinct chromatin arrangement in terms of the physical volume it occupies in space. Active regions occupied large volumes and became less densely packed as the genomic length increased. Instead inactive regions, although bigger in volume than repressed regions, occupied small volumes and had an increased packaging density with increasing genomic length [24]. This underlined the role of the PcG proteins to force chromatin compaction. While this study has been carried out in *Drosophila* cells, it opens novel ways to measure chromatin folding of specific chromatin domains during the processes of somatic cell reprogramming or differentiation.

Imaging living cells using single particle tracking provides key information on the dynamics of transcription factor activity

Single particle tracking (SPT) experiments have shown essential information on the dynamic activities of transcription factors and consequent gene regulation in different cellular contexts [25, 26]. An ordered kinetics in the binding of Sox2 and Oct4 in ESCs was reported using SPT imaging. Sox2 was seen to bind first and to prime target sites for Oct4 binding. However, Oct4 was seen just to help to stabilize the binding of Sox2 to the DNA. Remarkably, in this study with elegant computational work, the authors reported that these TFs spend the large majority of the time in performing trial and error approaches and undergo multiple rounds of nonspecific short chromatin binding events. Finally the TFs encounter a specific DNA target where they bind more stably. Upon treatment of

the ESCs with TSA or with the DNA methyltransferase inhibitor 5-AZA the number of accessible binding sites increase and the time of free 3D diffusion decreases. Intriguingly, the number of Sox2 molecules necessary to find the targets is calculated, suggesting specific TF concentrations are key for their correct binding [27].

Although the kinetics of TFs has been investigated in living ESCs it will be much informative in the future to observe the kinetics of TFs directly at endogenous genes by tracking them as they approach stem cell gene loci.

In another study, using lattice light sheet single molecule imaging, the positions of stable clustered Sox2 molecules were mapped. These were called Sox2 bound enhancers and formed highly density clusters [28]. By investigating the co-localization of these enhancer clusters with euchromatin (such as PolymeraseII) and heterochromatin (such as HP1) markers, Sox2 was shown to bind the enhancer clusters segregated from heterochromatin and naked DNA. Sox2 was seen to travel between clusters with a 3D diffusion mode, while in the clusters this 3D diffusion mode was shorter [28].

These observations are not fully concordant with the recognized pioneer role of Sox2 and therefore its ability to bind heterochromatin [29]. Perhaps one can speculate that during 4-factor induced reprogramming the overexpression of Sox2 along with the other three stem cell factors let it to become a pioneer factor and increase its binding to compacted chromatin.

Conclusions and future outlooks

Advanced microscopy methods have helped enormously to dissect the structure of the chromatin fiber in embryonic and adult stem cells. The understanding of the chromatin fiber conformation as well as the visualization of the dynamics of transcription factor binding has contributed to unveil gene activity and function. Until now, much work has been focused on cellular endpoint states, i.e. chromatin fiber structure in somatic, embryonic, fully reprogrammed, partially reprogrammed and in adult stem cells. Future efforts are necessary to study how the chromatin changes in the transition among all these states, thus at the onset and during the course of somatic cell reprogramming to pluripotency. In the next coming years the development of more robust methods to image single gene loci in living cells will allow enormous progress towards this direction. The expectation is to obtain quantitative data, which will help to identify the pluripotency grade of each cell starting by a novel way to analyze gene function.

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Annotated References

- special interest
- outstanding interest

[15]• Novo CL, Tang C, Ahmed K et al. The pluripotency factor Nanog regulates pericentromeric heterochromatin organization in mouse embryonic stem cells. *Genes Dev* 2016; 30:1101-1115.

By comparing Nanog^{-/-} mouse ESCs with wild type ESCs, the authors show that the pluripotency factor Nanog associates with heterochromatin domains and induces an open heterochromatin architecture. Down-regulation of Nanog, one of the first event during ESC differentiation is necessary for heterochromatin compaction. The study provides the first evidence of a direct link between a pluripotency transcription factor and higher-order chromatin organization in ESCs.

[18]• Ugarte F, Sousae R, Cinquin B et al. Progressive Chromatin Condensation and H3K9 Methylation Regulate the Differentiation of Embryonic and Hematopoietic Stem Cells. *Stem Cell Reports* 2015; 5:728-740.

By using nuclease sensitivity assay and Electron Spectroscopic Imaging (ESI) the authors show a progressive chromatin compaction increasing from unmanipulated ESCs, freshly isolated HSCs and their progeny. The amount of euchromatin was positively correlated with nuclear size and decreases upon differentiation condensing into highly packed heterochromatin which were also relocated to the nuclear periphery. The induced open chromatin state through G9a enzyme inhibition lead to a delay in differentiation.

[21]•• Ricci MA, Manzo C, Garcia-Parajo MF et al. Chromatin fibers are formed by heterogeneous groups of nucleosomes in vivo. *Cell* 2015; 160:1145-1158.

By using Stochastic Optical Reconstruction Microscopy (STORM) the authors show a novel chromatin organization model in pluripotent and differentiated cells. Nucleosomes are associated in clutches of different sizes and clutch size strongly correlates with the pluripotency grade of the cell. Ground state pluripotent stem cells have clutches with few nucleosomes compared to low-grade iPSCs and somatic cells. RNA Polymerase II associates to small clutches, while big clutches are enriched for the histone H1.

[24]•• Boettiger AN, Bintu B, Moffitt JR et al. Super-resolution imaging reveals distinct chromatin folding for different epigenetic states. *Nature* 2016; 529:418-422.

Using 3D-STORM and Oligopaint the authors investigated the 3D organization of several chromatin domains in *Drosophila* cells. They found a distinct chromatin folding of these domains classified as transcriptionally active, inactive or Polycomb-repressed, which was dependent on the epigenetic state. In this study for the first time the Oligopaint approach has been used to image with high resolution specific regulatory genomic regions.

Figure legends

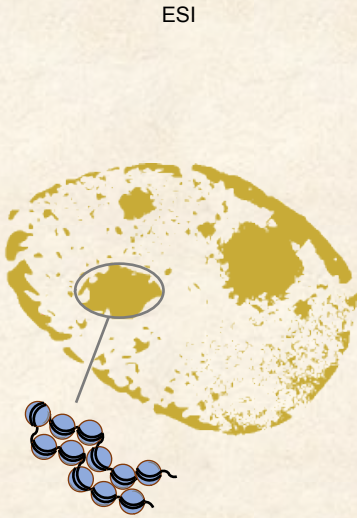
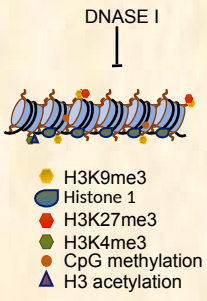
Figure 1. Visualizing and modeling chromatin structure using different advanced microscopy methods. Representation of the chromatin organization in somatic and pluripotent cells using different advanced microscopy methods. In the left, cartoon representing the epigenetic signatures of the closed and open chromatin states, typically associated with somatic and pluripotent cells respectively. In the central left, ESI Nitrogen map (yellow) of somatic cells (MEFs, Lymphocytes, Hepatocytes, upper nucleus) showed highly compacted chromatin regions at the periphery and in central areas of the nucleus. Example of an H3K9me3- enriched chromocenter is outlined in gray. Chromocenters are formed of 10 nm fibers and not of 30 nm fibers. In mESCs (bottom nucleus) and iPSCs the chromatin is more homogeneously dispersed in the all nucleus. In central right, 3D-SIM of DAPI-stained DNA of human hematopoietic progenitors (upper nucleus) and differentiated cells (bottom nucleus) showed a general architectural reorganization of the active and inactive nuclear compartments during differentiation. While somatic cells (granulocytes, upper nucleus) have a uniform layer of chromatin domain clusters (CDCs) at the nuclear periphery around a large DNA-free central lacuna (IC), the progenitors (bottom nucleus) have dispersed and decondensed chromatin regions between CDCs and the IC system. The active chromatin histone mark H3K4me3 has been localized within the decondensed chromatin layer, whereas SC35 protein is enriched within the IC. The RNA Polymerase II Ser2P has been found in an intermediate localization in both the IC system and in the less condensed chromatin regions. In the right, 2D-STORM of the histone protein H2B, revealed that the chromatin fiber organizes in a distinct way in somatic cells (human Fibroblast, upper nucleus) and in pluripotent stem cells (mESC, bottom nucleus). The chromatin fiber is formed by clutches of nucleosomes. Nucleosome clutches are bigger and denser in somatic cells and smaller and less packed in pluripotent stem cells. Big clutches mainly constitutes the heterochromatin regions and are enriched for the linker histone H1, while the smaller clutches are more closely associated to RNA Polymerase II.

Figure 2. Super resolution images of the chromatin structure. Histone H2B imaging with two different advanced super resolution methods. **A.** 3D-SIM light optical section of DAPI-stained DNA from HeLa cells. On the right, a representative magnification is shown. Image, courtesy of Pablo Hernandez-Varas, European Product Specialist, Nikon Instruments Europe BV. **B.** STORM 2D image of a human fibroblast nucleus stained for the histone protein H2B. On the right, a representative magnification is shown.

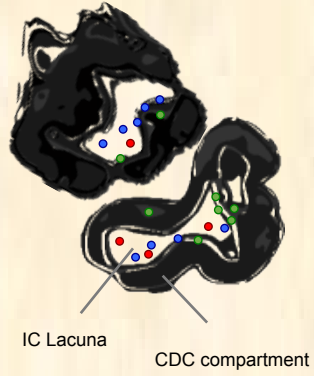
Figure 1

RESOLUTION
MOLECULAR SPECIFICITY

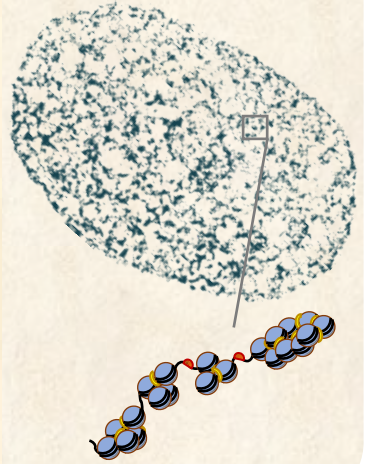
SOMATIC CELLS



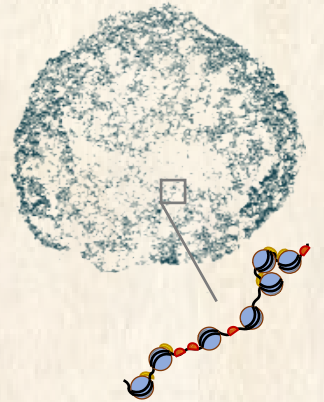
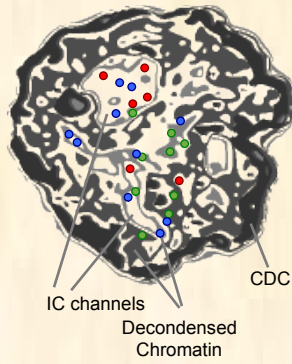
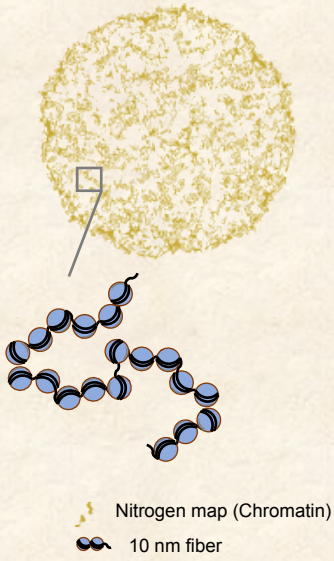
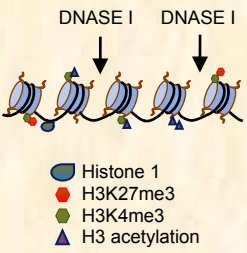
3D SIM



STORM



PLURIPOTENT CELLS



Chromatin (DAPI)

H3K4me3

RNA Polymerase II Ser 2

SC35

Histone protein H2B

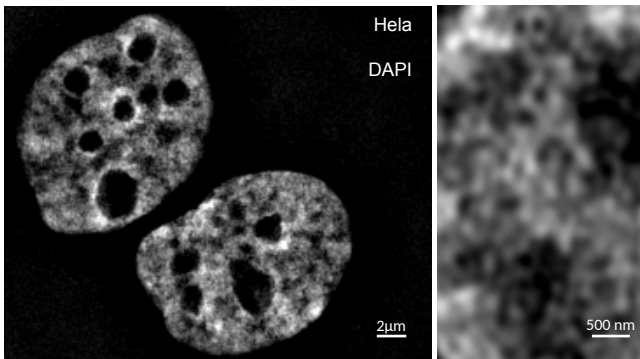
Nucleosome clutch

RNA Polymerase II

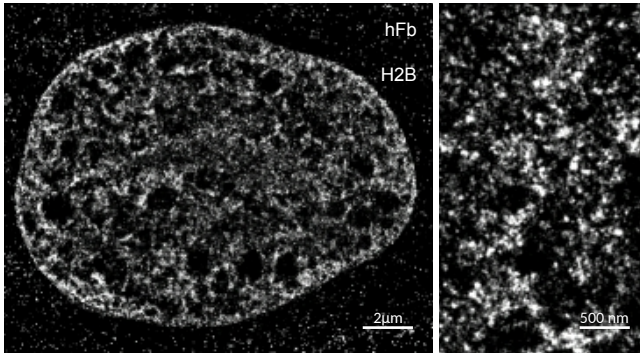
Histone linker H1

Figure 2

A Chromatin with 3D SIM



B Chromatin with STORM



Box 1. The advanced microscopy methods.

	Principle	Advantage	Disadvantage
Electron Spectroscopic Imaging (ESI)	As electrons pass through a thin specimen they can lose energy through inelastic scattering. The energy loss is used to extract information on the elemental composition of the specimen creating "spectroscopic" images.	Can discriminate between nucleic acids and protein content of chromatin [30]. Does not require heavy atom contrast agents.	Limited molecular specificity.
Soft X-Ray Tomography (SXT)	A beam of soft X-ray photons, used for illumination, is attenuated as it travels through the sample and the amount of attenuation is directly proportional to the type of molecular species and its concentration. Hence, Linear Absorption Coefficient (LAC) can be used to create quantitative tomographic images of different molecular species.	High resolution, quantitative imaging method that can be used for tomographic 3D imaging [31]. Cryo-preserved cells are imaged without the need for chemical fixation, sectioning or staining	Limited molecular specificity.
Light microscopy based Super Resolution Methods	Structured Illumination (SIM)	A sinusoidal pattern of bright and dark stripes is used to illuminate a fluorescently labelled sample, giving rise to "moiré fringes". Knowledge of the original illumination pattern can thus be used to extract the unknown sample features from these moiré fringes [32].	Low light levels and fast acquisition make it suitable for live cell imaging. High Molecular specificity.
	Stimulated Emission Depletion (STED)	A doughnut shaped depletion beam is used to force the molecules lying in the doughnut area back into the ground state through stimulated emission. The fluorescence signal from these fluorophores is then filtered, effectively leading to switching off of these fluorophores and a reduction in the excitation volume [33, 34].	High spatial resolution (~20-50 nm in 3D) [35]. High Molecular specificity.
	Single Molecule Localization Microscopy (SMLM): PALM STORM PAINT	Photoswitchable fluorescent probes are used to reduce the number of fluorophores in the bright state at a given time and avoid overlap of fluorescent signal coming from individual fluorophores. In this regime, the image of each fluorophore can be identified and localized with high precision. An image is then reconstructed by localizing several fluorophores over time [36-40].	High spatial resolution (~20-50 nm in 3D) [41-44]. High Molecular specificity [45, 46].

Inclusion of Highlights do not apply to the Review.