Cryo-nanoscale chromosome imaging –future prospects

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Abstract

The high order structure of mitotic chromosomes remains to be fully elucidated. How nucleosomes compact at various structural levels into a condensed mitotic chromosome is unclear. Cryogenic preservation and imaging have been applied for over three decades, keeping biological structures close to the native *in vivo* state. Despite being extensively utilized, this field is still wide open for mitotic chromosome research. In this review, we focus specifically on cryogenic efforts for determining the mitotic nanoscale chromatin structures. We describe vitrification methods, current status, and applications of advanced cryo microscopy including future tools required for resolving the native architecture of these fascinating structures that hold the instructions to life.

Keywords: mitotic, chromosome, chromatin, nanoscale, cryo, freezing, microscopy

Introduction

Chromosomes were first discovered over a century ago[1] with microscopy having been applied for decades to image these fascinating structures that hold the instructions for life. The basic building block of mitotic chromosomes is chromatin, the DNA-protein complex composed of discrete nucleosomes, which can be thought of as the "atoms" of the chromosome structure. To date, our understanding of how these nucleosomes are organized into highly compact chromosomes so-called the 'high-order structure' is largely unknown. Approximately 2 meters of DNA is packaged inside each cell nucleus that is approximately 10 μM in diameter.[2] Nucleosome compaction and de-compaction plays an essential role in packaging DNA during vital biological processes including transcription, gene expression, DNA replication, faithful separation and repair.[3-6] The most compact state of chromatin occurs in the mitotic state of the cell cycle, in which chromosomes condense before cell division and then segregate into identical daughter cell copies after significant conformational changes.[7,8] This mechanism involves the formation of chromatin loops that tether the axial structure of the chromosome.[8] For understanding how chromatin is organized into mitotic chromosomes, several different experimental approaches have been explored including sequencing,[9] chromosome conformation capture that includes HiC[10,11] and direct imaging.[12,13,14]

A range of imaging technologies have been applied for nanoscale elucidation of chromatin at various compaction states ranging from the 2 nm thick DNA fiber to the compact mitotic

chromosome.[13] Advanced microscopy using X-rays revealed the nucleosome's crystal structure consisting of DNA (145-147 bp) wrapped approximately 1.65 times around eight histone proteins (core histone octamer composed of H2A, H2B, H3 and H4).[15,16] This nucleosome-histone octamer exhibits a 11 nm diameter bead on a string like structure observed by electron microscopy (EM) *in vitro,*[17] with H1 histone involved in linking and directing compaction of nucleosomes.[18,19] Cryogenic electron microscopy (cryoEM) showed the linker DNA to have a stem-like organization upon entering and exiting the nucleosome particle.[20] The tetra nucleosome structure has also been resolved using x-rays,[21] EM,[22] and cryoEM.[23] Further, this 11 nm bead on a string like structure gives rise to the controversial high order 30 nm structure that is the most debated topic in the field,[24-28] with many models proposed.[14,26,29,30] CryoEM has shown an interdigitated one-start solenoid model displaying 30 nm fibers[31] whereas other cryo studies do not favor this structure.[32] Apart from nucleosomes being the building blocks, the overall mitotic chromosome compaction is dependent on numerous proteins with over 158 been identified[33,34] that include the non-histone scaffold proteins such as cohesion, condensin and topoisomerases.[35] The various stages of chromatin structure condensation is partly understood with several factors implicated.[25,35,36,37]

Imaging chromatin structures close to the native state at nanoscopic resolution is vital for understanding the architecture of these compact and complex structures. In this mini-review, we summarize our current knowledge of higher-order mitotic chromosome structure from chemically preserved samples. We then focus on the need of cryo technology for mitotic chromatin nanoscale structure determination. We highlight vitrification methods including cryo microscopy studies used for unraveling the high order structure of intact mitotic chromosomes. Further, we discuss current limitations and the future need for cryo technology for elucidating the high order hierarchy of these fascinating structures.

Lessons from fixed chromosome samples

1n 1956, the correct number of human diploid chromosomes (46) was identified[38] utilizing standard optical microscopy at its highest resolution of 200 nm.[39] To unravel the nanoscale chromatin structure, advanced microscopy has been applied, revolutionizing our understanding of chromosome structure.[40] Even though useful structural information was provided, these studies heavily relied on preserving/fixing samples using harsh chemicals, mainly aldehydes, dehydration

steps and staining. Such harsh conditions influence the native sample configuration and hence what is observed is a reproducible artifact not the actual native state.[41,42] These chemically preserved chromosome samples have been imaged using different ultra-high-resolution microscopes that are mentioned below and summarized in Table 1.

Super resolution microscopy (SRM), an advanced fluorescence imaging method provides high resolution (~10 to 30 nm) [43-45] and has been extensively used for interphase chromatin structures.[46-47] SRM to a lesser extent has been explored for unraveling mitotic chromatin structures. Stimulated emission depletion (STED) SRM on fixed HeLa cells showed sub-chromosomal localization of immuno-stained condensin II subunit CAP-H2 protein along the length of mitotic chromosomes being enriched mainly around the longitudinal chromatid axis.[48] Three-dimensional structured illumination microscopy (3D-SIM) together with focused ion beam (FIB) milling EM done on HeLa cells showed distribution of chromosome scaffold proteins condensins and topoisomerase Iiα having two main lateral strands twisted around each other within the chromatid axis.[49]

Single molecule localization microscopy (SMLM) using a modified Spectral Position Determination Microscopy (SPDM) approach[50] displayed a mean localization accuracy around 14 nm from high-quality DNA density maps of mitotic HeLa chromosomes after staining with Hoechst 33342.[51] DNA single-molecule Photo-activated Localization Microscopy (PALM) showed sub 20 nm structure resolution in unstained HeLa chromosomes with nucleotide density variations and fine features on chromatids.[52] PALM revealed ~70 nm structures composed of 35-nm stripes on *Drosophila* mitotic chromosomes after labeling with H2AvD-EGFP, a histone H2A variant.[53]

EM is a contrast imaging method that provides detail at high resolution.[54] Scanning electron microscopy (SEM) has provided surface information on B-lymphocyte chromosomes showing chromatin fibers ranging between of 25-35 nm diameters.[55,56] Often a nucleoplasm layer is seen on the surface of the chromosomes that hinders high resolution chromatin substructure determination[56] This has been overcome by applying 3D serial block face SEM (SBFSEM) that embeds the sample into resin followed by automated diamond knife sectioning providing or resulting in nanoscale resolution of B-lymphocyte chromosomes that were isolated[57] and within a prophase nucleus.[58]This study showed porous network structures on chromosomes with sister chromatids having conserved diameters of around 765 nm.[58] An alternative method to SBFSEM

is focused ion beam SEM (FIBSEM) that uses an ion beam for sectioning and has provided structural information of barley chromosomes[59] Centromeres displayed parallel fibrils whereas both chromosome arms (p and q) showed extended cavities also known as chromomeres [59].

Transmission electron microscopy (TEM) provides angstrom resolution but is not suitable for studying intact mitotic chromosomes due to their thickness.[60] Studies on chromatin using TEM have been explored in 2D extensively and are reviewed elsewhere[61]. 3D TEM tomography has been applied on chemically flattened HeLa chromosomes that revealed 26 to 58 nm structures displaying a 30 nm fiber distribution.[62] ChromEM tomography (ChromEMT) a multitilt EM tomography and a labelling method showed irregular disordered chains of nucleosomes with 5 and 24 nm diameters, indicating that the 10 nm fiber is heterogeneous.[63] A helical structure composed of chromatin loops was detected when the CAP-E protein, a condensin subunit, was labeled with gold nanoparticles after the chromosome was isolated by FIB and imaged using ET.[64]

Atomic force microscopy (AFM) provides atomic resolution with surface topology information and has been applied for determining chromosomal detailed structures and reviewed in detail elsewhere[65-67]. Giemsa (G) banded chromosomes displayed ridges and grooves corresponding to heterochromatin (dark) and euchromatin (light) bands, respectively. This technique revealed highly twisted chromatin fiber loops with stronger compaction in the ridged regions than grooved. Additionally granular and/or fibrous 50-60 nm structures were also seen on the surface[67].

Mitotic chromosomes have also been imaged at nanoscale resolution using x-rays that can penetrate the sample with no sectioning needed[68] as they have a shorter wavelength[69]. Soft x-ray displayed 70 nm resolution showing varied chromosome thicknesses ranging from 150 to 750 nm from metaphase to anaphase, respectively.[70] A pioneering hard x-ray diffraction study displayed 30 nm and 120 nm structures in 2D and 3D, respectively of unstained intact mitotic chromosomes.[71]

Cryo preservation methods suitable for mitotic chromosomes

Cryobiology refers to biological samples subjected to temperatures below their normal range.[72] Imaging biological samples near-native state is achieved after vitrifying the biological sample in amorphous ice, known as cryopreservation, cryofixation or cryo-immobilization.[73-75] This allows

instant fixation of all molecules present in the sample that remain at the set position displaying a true representation of the sample at the given point of freezing.[76] For vitrification, the sample temperature has to remain below −140°C with freezing that should occur rapidly within microseconds at high rates (~10^4 °C/s or higher).[77,78] Ice crystal growth is prevented in this procedure allowing macromolecule immobilization and allowing the specimen to be in an amorphous (close to native) state.[79,80] The in laboratory procedure involves rapid plunging (plunge freezing) into liquid ethane or propane after the sample is prepared on EM grids and blotted[83] but is only useful for vitrifying thin samples below 1 μm.[78,84] Slam freezing is also an alternative but can be used for samples up to 10 μm thickness[78] Thicker volume samples (200 μm and larger) require high pressure freezing that uses pressure of approx. 2100 atmospheres (or bar) for a few milliseconds in order to freeze the sample with no ice crystal damage.[85,86] Thicker biological samples after freezing can be cryosectioned using cryo-ultramicrotomy and is a method referred to as cryoelectron microscopy of vitreous sections (CEMOVIS).[41] Obtaining thin sections (40–100 nm) prior to imaging is extremely challenging as well as time consuming.[41] To image samples at room temperature, freeze substitution can be performed where the sample (water) is replaced using organic solvents (acetone or methanol) at low temperatures (approx. -78° to -90°C). In many cases, osmium tetroxide or glutaraldehyde fixatives are included in the acetone to provide fixation that is achieved by raising the temperature slowly (typically 5°–10°C/h) in order to achieve the reaction.[87] These sample are then placed into resin after staining and sectioned at room temperature before imaging.[88] Again these procedures are technically challenging, time consuming, can cause artifacts and are costly (Figure 1).

Cryo imaging of mitotic chromosomes

Cryo-EM serves as a useful label free approach for imaging frozen hydrated biological samples close to native state.[89,90] The cryo-electron tomography (cryo-ET) known as the 2016 "Method of the Year"[91] was later awarded the 2017 Nobel prize for cryo-EM development including determination of high-resolution biomolecule structures in solution.[92] cryo-ET provides 3D position of the sample inside cells at ~4-nm resolution.[93] Both cryoEM and cryo-ET have enhanced our understanding in resolving atomic structures of the nucleosome[94-95] and interphase chromatin organization, [96-99] respectively. The number of microscopy studies on mitotic nanoscale structure under cryo conditions has been limited (Figure 1).

CryoEM/ET cannot be applied directly for mitotic chromosome imaging as this technology requires the thickness of the sample to be below 1 μM. [100,101] Therefore the CEMOVIS method.[41] has proven critical for mitotic chromosomes. The CEMOVIS method after 100-150 nm sections has been applied directly on unstained Chinese hamster ovary (CHO) and HeLa cells with 11 nm chromatin filaments.[102] 30 nm fibers were not seen in 40 nm cryosections of chromosomes within mitotic HeLa cells, instead highly disordered and interdigitated structures were visulised.[96] Compact stacked multilaminated plates were seen after applying CryoEM on HeLa and chicken lymphocyte cells that orientated perpendicular to the chromatid axis.[103] A recent 3D cryoTEM study that used HeLa cells showed that frozen hydrated DNA is densely packed, forming stacked sheets of chromatin, is planar and forms multilaminar plates that are stabilized by interactions between nucleosomes. Having a 13 nm thickness between the two layers (single layer 7.5 nm) implicated that nucleosomes in the layers interdigitate. Together with small angle X-ray scattering (SAXS) data, a chromosome model was proposed composed of stacked chromatin layers positioned perpendicular to the axis of the chromosome.[104]

Mitotic chromatin in frozen-hydrated *Schizosaccharomyces pombe* displayed megacomplexes and pockets, showing more compaction at the oligo-nucleosome than the di-nucleosome level compared to interphase chromatin.[105] Mitotic chromatin organization showed no evidence of 30 nm fibers in budding yeast *Saccharomyces cerevisiae*[106] and picoplankton.[107] A X-ray cryo-ptychography experiment has been attempted on human chromosomes but required more work in optimizing the setup before any concrete conclusion could be made.[108]

What next? Is there a need for Cryo?

Even though cryo imaging has proven useful for numerous chromatin-based studies including complexes, it has not been fully exploited for intact mitotic chromosome investigation**.** Freezing of chromosomes can be achieved using the different vitrification methods but chemical preservation or freeze substitution will be the only option if the microscope of choice does not have cryo capability for sample cooling during imaging. Current high-throughput structure determination has been prevented due to several limitations that include: i) thickness of the compact mitotic chromosomes; ii) challenging cryo sample preparation steps, handling and

preservation; iii) powerful nanometer cryo imaging microscopes with sufficient resolution, and iv) computational tools for image acquisition and detailed processing.

The number of studies done on mitotic chromosomes has not been fully exploited using cryo technology. However we are now witnessing an increase in the number of studies (see timeline) using advanced cryo technologies that recapitulate the close to native state of mitotic chromatin structure. The CEMOVIS method[41] is so far the widely explored on mitotic chromosomes (see timeline). No interspecies structural variation was observed for the presence of the 30-nm chromatin structure apart from one study that occasionally showed this on HeLa chromosomes. [104]HeLa is a cancer cell line[109] and has been widely used for determining human nanoscale chromatin structures in both chemical preserved (Table 1) and cryo studies (Figure 1). HeLa cells are extremely complex and heterogeneous and display abnormal karyotypes[110,111] that can add to the variability reported in current imaging studies and may not represent the 'true 'picture'.

We are now witnessing various imaging approaches that are enhancing our knowledge in cryo imaging of biological samples but are yet to be explored for mitotic chromosomes. SBFSEM does not yet have a cryogenic stage for the instrument therefore the samples have to be imaged at room temperature after resin embedding.[57] Therefore freeze substitution of chromosomes after high pressure freezing and SBFSEM would be a positive way forward.[112] Alternatively cryo-FIB that has full cryo capability[113] would be useful providing close to native state imaging and is yet to be experimented directly on vitrified chromosomes. Another potential method that needs exploring is STORM SRM under cryogenic conditions as this using a allowed 12 nm resolution to be achieved on bacterial cells using a low-cost super-hemispherical solid immersion lens (*super*SIL).[114,115]

A combination of microcopy approaches is also being used and looks promising[113]. 3D CLEM performed using light and SBFSEM has been performed to understand the role of Ki-67 in metaphase chromosomes at ultra-structural resolution.[116] This correlative technology using with 3D SIM, SMLM and FIB-SEM has been applied on mammalian cells after combining vitreous freezing and is known as cryoCLEM.[112] This SRM combined with FIBSEM may serve useful for DNA/protein structural interactions on mitotic chromosomes.

As cryo preservation only allows a snapshot of a biological process at a single time point therefore it is key to trace dynamic chromatin movement *in vivo*. SRM using PALM and tracking of live cells has shown ~140 nm and ~200 nm mitotic chromatin domain that were suggested to be

retained throughout the cell cycle.[118] Furthermore new microfluidic based technology allows direct correlation of live imaging and room-temperature electron microscopy with millisecond time resolution after the sample is cryofixed.[119] Recently, this technology has been combined with cryo-FIB to prepare frozen hydrated electron transparent sections for cryo-ET.[120] This powerful 4D high resolution space-time correlative light and electron microscopy (st-CLEM) method is useful but needs to be explored on mitotic chromosomes. The use of DNA painting that allows blinking after binding of short dye-labeled ('imager') oligonucleotides to their complementary target ('docking') strands is serving useful for chromosome nanoscale SRM imaging.[121,122]

The future of chromosome imaging without doubt is moving towards full cryogenic settings that will be crucial for answering fundamental biological questions in the chromosome field. We must consider mitotic chromatin complexity from current studies (Figure 1, Table 1) considering variations in different organisms, developmental stages including pluripotency and epigenetic states, cell types (undifferentiated vs differentiated), cell cycle stages (interphase vs metaphase), chromosome types ((sub)metacentric/acrocentric), compaction states (g-bands) e.g. heterochromatin vs euchromatin and telomeres/centromeres. Furthermore cryo imaging has been performed both on isolated chromosomes and on chromosomes inside a cell after performing the CEMOVIS/cryoEM method (Figure 1) indicating no major structural variability seen so far and this would have to be carefully considered for future studies. Powerful and affordable 3D cryo-microscopes with nanoscale resolution together with faster image processing tools, correct sample and labeling choice will be essential for unravelling nucleosome-nucleosome with other protein/DNA interactions. Overall this would assist in identifying disease specific signatures relating to genome disorganization especially in cancer where chromosomal aberrations take place.

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Conflicts of interest

There are no conflicts to declare.

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Microscope	Species	Cell type	Isolated chromosomes or intact cells	Staining	Findings	Publication
STED	Human	HeLa	Intact Cells	mEGFP-CAP-H2	Condensin II subunit CAP-H2 protein enriched around longitudinal chromatid axis	48
SPDM (SMLM)	Human	Hela	isolated	Hoechst 33342	Density maps displaying a mean localization accuracy around 14 nm	50
PLM (SMLM)	Human	HeLa	isolated	auto fluorescence	Nucleotide density variation in chromatids and fragile site like features	52
SIM	Human	Hela	isolated	anti-Topo Iiα, anti-histone H3, anti-hCAP-E, anti-KIF4A	Two main lateral strands with a twisted axial distribution of scaffold proteins (FIB included)	49
PALM	Drosophila	Embryo	cell	H2AvD-EGFP	70-nm filamentous blocks composed of stripes of 35-nm sub-filaments	53
SEM	Human	Lymphocytes	isolated	Metal coated	Irregular, twisted and entangled 25- 35 nm chromatin fibers on chromatids	55
SEM	Human	B-lymphocyte	isolated	platinum-based dye	Globule chromatin size between 15 and 30 nm, with characteristic diameter of around 20nm	56
SBFSEM	Human	B-lymphocyte	isolated	platinum blue	Internal structural cavities seen using MAA samples only	57
SBFSEM	Human	B-lymphocyte	Inside cells	platinum blue	Porous network structure on chromosome arms with 50 nm resolution in 3D	58
FIBSEM	Barley	Seeds -*Hordeum vulgare*	Isolated	platinum blue immunogold labeled for phosphorylated histone H3	Parallel fibrils seen at centromere and extended cavities on chromosome arms Strong labeling in the pericentric regions with a signal at the centromere	59
TEM	Human	HeLa	isolated	uranyl acetate	Chromosome fibers of 25–60nm are seen	62
TEM	Human	Primary human small airway epithelial and U2OS cells	Intact cells	DRAQ5 followed by osmium tetroxide	Chromatin forms flexible chains with diameters between 5 and 24 nm	63
TEM	Human	HeLa	isolated	ionic liquid anti-CAP-E , Fluoronanogold	CAP-E observed at central axis in each chromatid and diffused in arms displaying helical structure	64
AFM	Human	Lymphocytes B-ALL-1	isolated	Giemsa and metal staining	Structure of chromatid arm not uniform. ridges and grooves seen that correspond to G-positive and G-negative bands	67
X-ray	Human	B-lymphocyte	Isolated	platinum blue	Internal fibrous ultrastructurs observed	68
X-ray	Human	Fibroblast cell line, NIH3T3 cells	isolated	-	Thickness of the chromosome varied from 150nm to 750nm	70
X-ray	Human	HeLa	isolated	-	Chromosome axial structure determined using both 2D and 3D	71

Abbreviations

SMLM - Single molecule localization microscopy
STED - Stimulated emission depletion
SPDM - Spectral Position Determination Microscopy
PLM - Photon localization microscopy
SIM – Structured illumination microscopy
PALM - Photoactivated Localization Microscopy
SEM - Scanning electron microscopy
SBFSEM - 3D serial block face SEM
FIBSEM - focused ion beam SEM
TEM - Transmission electron microscopy
AFM - Atomic force microscopy
HeLa - Human epithelial cells
U2OS - Human osteosarcoma cells
B-ALL-1 - B-cell acute lymphoblastic leukemia
NIH3T3 - immortalized mouse embryonic fibroblast cell line.

Table 1. Comparison of microscopy studies that used chemical preservation for investigating chromatin structure

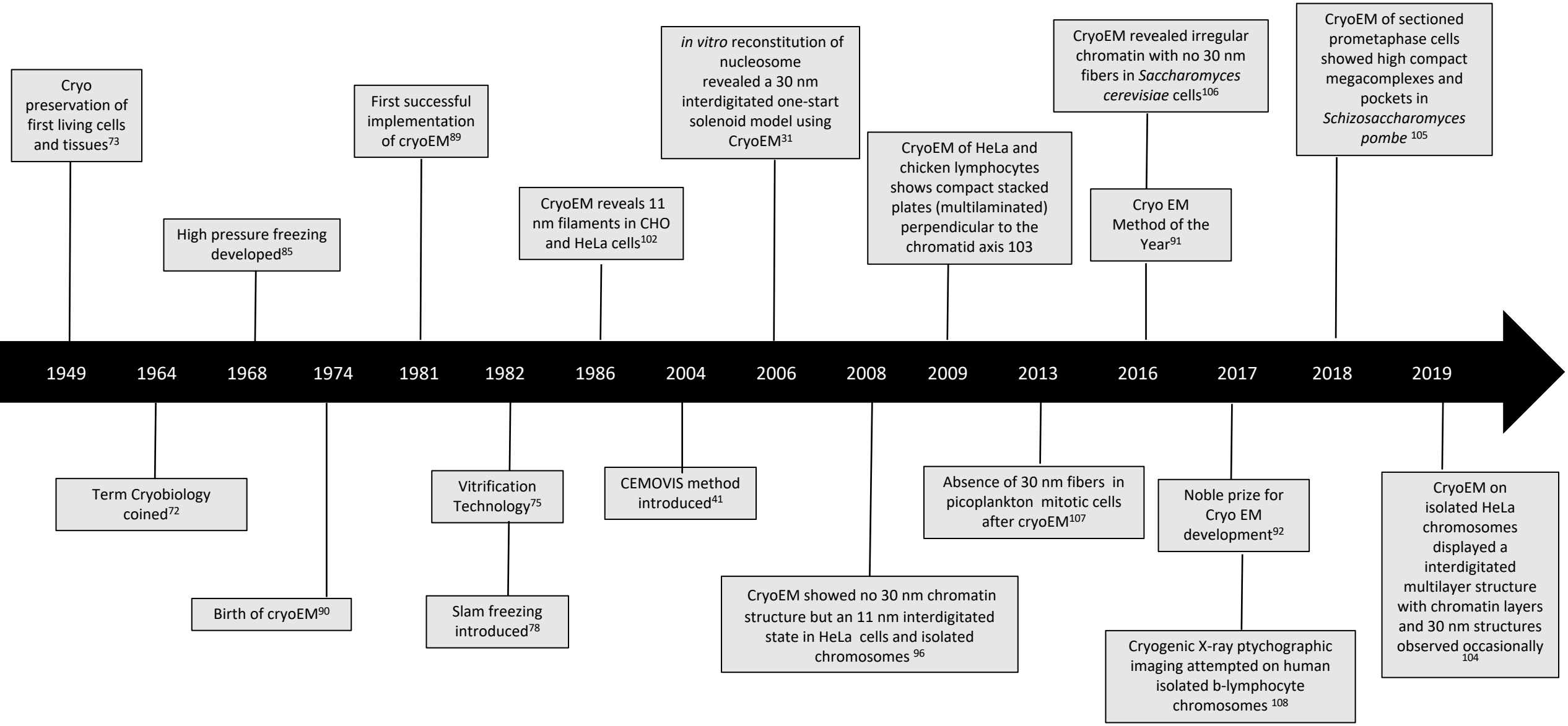

Figure 1 : Timeline. key events in the development of cryo preservation methods and imaging of mitotic nanoscale chromatin structures