Sample preparation of human chromosomes for cryo-X-ray imaging at I131

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Abstract

Sample preparation procedures were developed for imaging human chromosomes under cryo conditions using a new beamline cryo setup at I131. Human chromosomes were carefully plunge frozen to obtain vitrified samples. These samples were stored and transferred into a unique sample holder that was then used on the I131 beamline. This 8 week report discusses the sample preparation procedures and steps taken from preparing the chromosomes to imaging the sample using X-rays.

Keywords: Human Chromosomes, Sample Preparation, Chromosome Imaging, Cryo setup and I131beamline.

1.

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1. Introduction

Chromosomes are studied in clinical labs prepared from peripheral blood lymphocytes, bone marrow cells etc for validated stages of diseases. Chromatin (DNA and Proteins) are generally present in nucleated cell in thread-like structure during interphase (long part of cell cycle) and slowly get condensed during cell division at subsequent stages (Mitosis). It is more frequently studied at prophase/metaphase phase stage of cell cycle where they are in their most compact state. Various stains are used to study metaphase chromosomes with light, fluorescence, electron microscope and x-ray's (1).

1.1 Structure of Chromosomes

Chromatin is made up of repeated units of monomer-nucleosomes and nucleosomes consist of 8 histone proteins and 146 base pair of DNA (Deoxyribonucleic acid) strand. Chromosomes at their most compact state consist of a centromere that is an restriction site which divides chromosomes into two arms P-arm & Q-arm (depending on which chromosome sit is. It also consists of telomeres at the end of the chromosomes and various replication sites for the duplication of DNA prior to mitosis.

The intermediate structure of chromosomes is still unknown, researchers are still working to study the most controversial 30nm structure (2) if it actually exists or not.

The aim of this experiment was to study the structure of chromosomes after chromosomes were placed into vitrified ice by plunge freezing. Chemical methods disturb the native state of chromosomes; therefore freezing to retain the structure of chromosomes with high pressure freezing maintains the native structure of chromosomes (3).

1.2 Plunge Freezing

Aqueous biological high pressure plunge freezing maintains the native state of biological samples for imaging methods such as Cryo TEM and Cryo X-ray's. . In plunge freezing samples get vitrified in liquid nitrogen and become amorphous while plunging. Samples are stored in liquid nitrogen after plunge freezing at very low temperature. The advantages of plunge freezing is that it preserve the sample and gives high resolution images with less radiation damage but negative part of this procedure is that sample needs to be stained to get better contrast because unstained sample does not show good contrast and it is also limited to depth freezing.

2. Materials and Methods

2.1 Media Preparation

100ml of Fetal Bovine Serum (FBS);(2x45ml RPMI-1640), 5ml of Penicillin (100U/ml) streptomycin(100μg/ml), and 5ml of L-Glutamine(2mM) were mixed with RPMI medium and incubated at 37°C before use. All reagents were sprayed with 70% ethanol and brought inside a Laminar Air Flow (LAF). The media was mixed well, stored in 4°C fridge before use.

2.2 Storage of Cells in Liquid Nitrogen

Cells were stored in cryotubes inside the dewar containing liquid nitrogen at -196°C to preserve and keep cells in their native state. During cell culture these cryotubes are thawed in water bath (37°C) prior to use.

2.3 Cell Culture

Once cells are taken from the liquid nitrogen, thawed cryotubes and cells were (1ml) transferred to a small cell culture flask, few millilitre fresh media added under LAF and incubated at 37°C for 2-3 days to get good growth of cells. After 2 days, cells were transferred from a T25 flask to a T75 and allow growing, cells transferred to T175 flask, left to grow subsequently splitting. The density of cells was obtained by performing a cell count. 10μl of the sample (media containing cells) plus 10μl of Trypan blue dye (0.4% life technologies) were mixed in one eppendorf. 10μl were placed into the cell counting slide (BIO-RAD), and a measurement was taken.

2.4 Chromosomes Preparation

3ml thymidine (0.045gms/3ml PBS (15mg/ml), Sigma)(4) was pipetted into flask conatining150 ml media growing cells. After 17 hours of thymidine treatment, cells were transferred into 50 ml falcon tubes, centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded into Virkon solution and the cells were washed in PBS (pH-7.4(1X), Phosphate Buffered Saline, Gibco, Life technologies). The cells in the falcon tube were centrifuged again at 1200 rpm for 10 minutes and added into fresh media. The cells were left to grow for 5 hours; 700 μl of colcemid (Karyomax, Gibco by Life technologies (10μg/ml) was added to 100 ml of media to arrest the cells at prophase/metaphase. An Overnight colcemid treatment was done; cells were transferred into 50 ml falcon tubes, spun at 1200 rpm for 10 minutes. The supernatant was discarded; the pellet was resuspended in PBS and centrifuged again at 1200 rpm for

10 minutes. The PBS supernatant was discarded and KCL (0.075 mM) was added and left for 5 mins treatment at 37 degrees. The tube was centrifuged again after 5 mins treatment at 1200 rpm for 10mins. The supernatant was discarded; first Methanol Acetic acid (MAA-75% methanol+25% acetic acid, 3:1) was added drop by drop and tapped fast to dislodge the pellet, spun at 1200 rpm for 10 minutes, supernatant was discarded. Fixation step (MAA-fixative) was repeated 2 times and the samples were stored at -20°C.

2.4.1 Spreading

Approx. 15 µl of chromosome sample was dropped on to a glass slide from a height to obtain good spreads of chromosomes. Once the sample dried it was stained with DAPI (4′, 6-Diamidine-2′-phenylindole dihydrochloride, Sigma, H-1200), covered with 1, 22 x 50 cover slip. The sample was observed using a fluorescence microscope (Zeiss Z2 Axio imager with Isis software). A 10x objectives was used to locate the chromosomes and observe sample density. A 63x objective (with oil) was used to see clear view of DAPI stained chromosomes. An Olympus laser scanning confocal microscope was also used to validate chromosomes spreads using Lext software. 5x objectives was used for localisation of chromosomes and 10x, 20x, 50x & 100x objectives were used for high resolution of chromosome spreads.

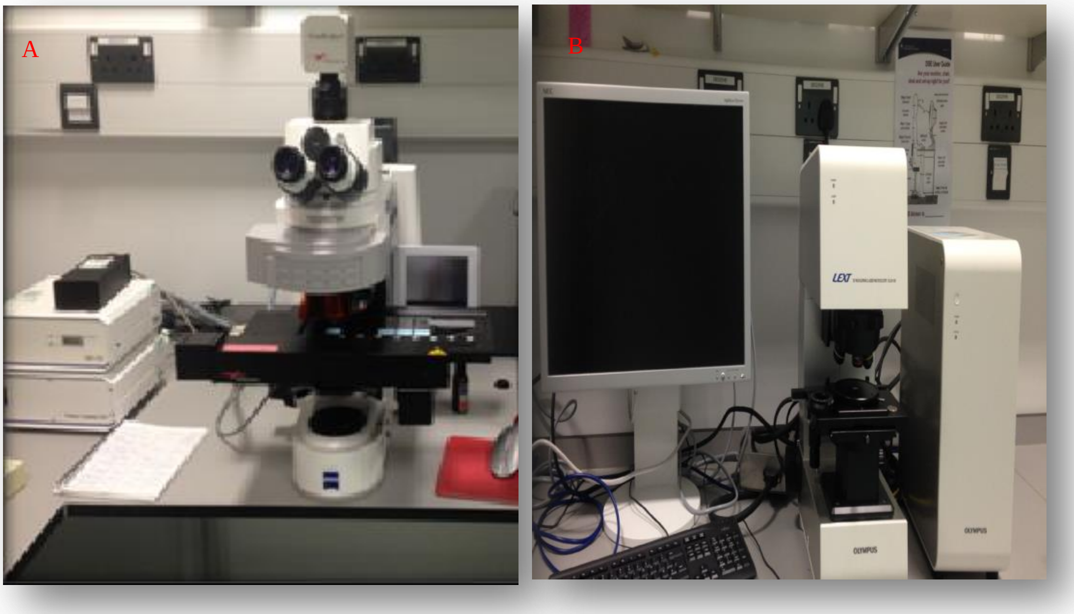

Figure 1: Microscope used for human chromosome observation, A) Fluorescence Microscope with MMC stage and B) Laser Confocal Microscope

2.5 Silicon Nitride and Quantifoils Grids

Throughout these experiment two types of TEM (Transmission Electron Microscope) supports were used i) octagon silicon nitride (Silson Ltd.) with 200 µm frame thickness, square shape 0.25 x 0.25mm membrane size & ii) carbon-coated 7 x 7 quantifoils with 400 mesh grids (Taab Laboratory Equipment Ltd.).

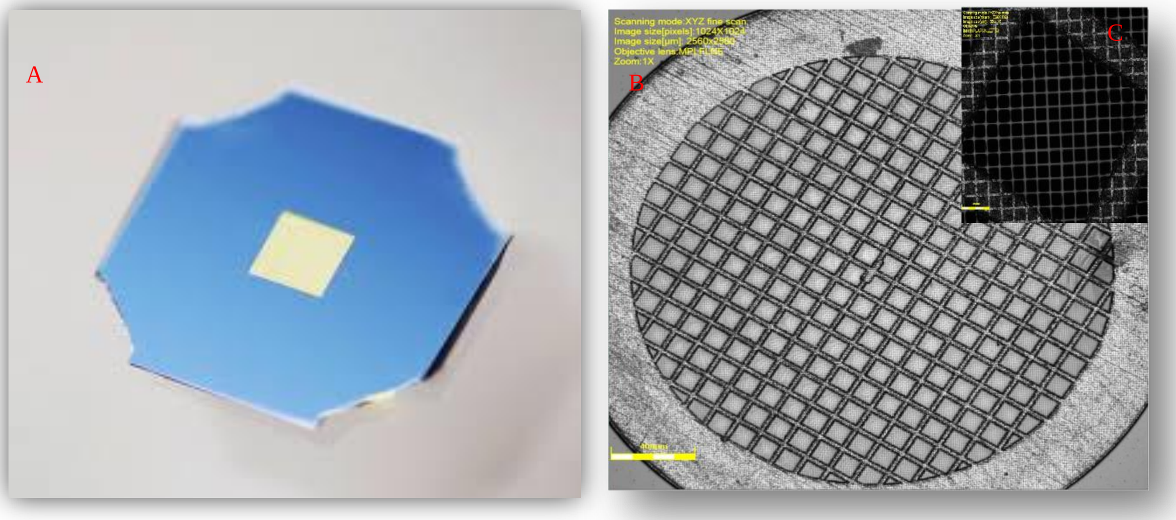

Figure 2: A) Silicon nitride grid with square shape inner membrane. B) Carbon-coated quantifoils with inner bar & mesh, scale bar-400µm. C) 7x7 inner grid of quantifoil and scale bar-20µm.

2.6 Hydrophilic Methods

Grids were made hydrophilic using 2 methods on the shiny side down of the grid, i) evacuated chamber connected with power supply using Quarum glow discharge equipment for 90 second was used ii) Poly-L-Lysine (1:10 dilution, Boster), treatment for 30 minutes.

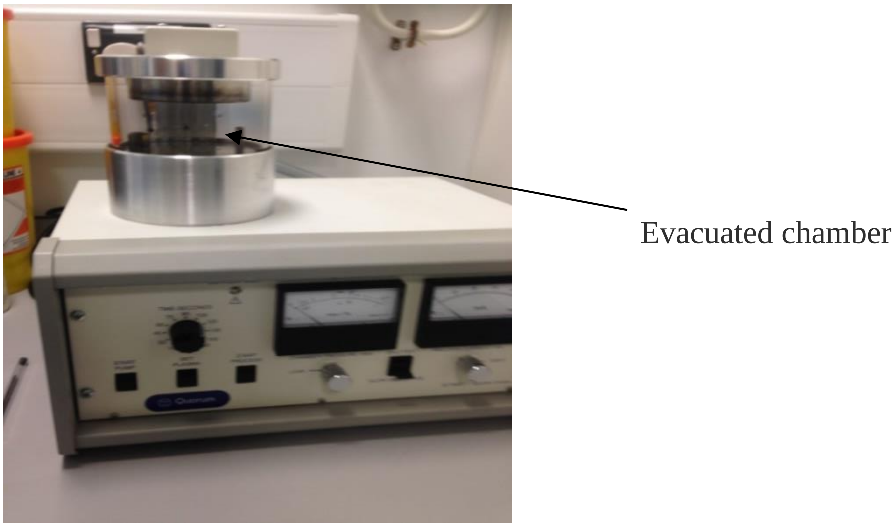

Figure 3: Quarum glow discharged equipment containing evacuated chamber.

2.7 Uranyl Acetate (UA) Staining for TEM

This is a negative stain which is done using a heavy metal called Uranium metal salt. 5µl/10µl of prepared chromosome was dropped on hydrophilic grids. Double staining of 2% aqueous uranyl acetate was added (5µl) on the sample grid, blotted for 1 minute before TEM (Joel) visualisation.

200nm gold nanoparticles (Sigma, Aldrich) were added onto the grids as test objects.

2.8 Plunge Freezing

Chromosome sample were added to the hydrophilic (glow discharged (7 x 7 copper grids) and left for 5 minutes before plunging. Some samples were stained with 5 mM platinum blue and washed with water and blotted before plunging. Samples were held with a fine tweezer and brought to plunger (Vitrobot figure 4 (B), where it again blotted for 1-2 seconds straight after blotting. The plunger dropped the sample into a cryogen tank (figure 4 (A), containing liquid nitrogen and liquid ethane, once the grid enters the liquid ethane, the sample is rapidly frozen and gets vitrified. Same procedure was repeated with all grids. The sample was stored in liquid nitrogen for later use.

Figure 4: A) Cryogen tank containing liquid nitrogen and ethane, where samples are vitrified. B) Plunger by which samples are blotted and dropped in cryogen tank.

2.9 Sample Transfer Stage to X-ray Beam

Chromosome sample stored in liquid nitrogen were brought into a foam dewar, containing liquid nitrogen. The brass transfer bucket and the sample holder were placed into the bath. Both the disc & sample holder were unscrewed under liquid nitrogen conditions. The quantifoil/silicon nitride windows were containing the sample and were transferred carefully from the disc to sample holder using fine tweezers. After screwing up the holder it was transferred to the brass bucket, which was then taken to the cryo work station.

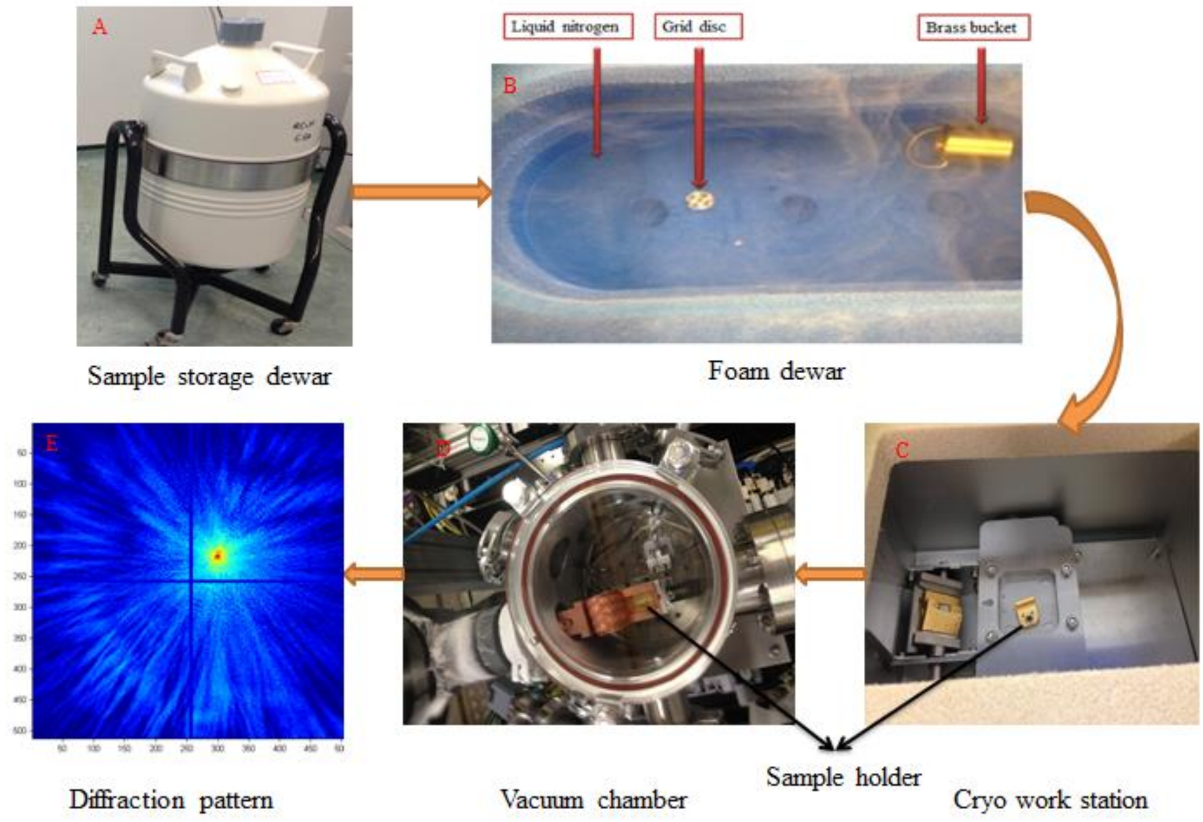

Figure 5: Flow diagram shows stages of human chromosomes sample transfer. A) Liquid nitrogen dewar where samples was stored, B) Foam dewar where all tricky transfer was done from grids disc to bucket under liquid nitrogen, C) Cryo work station- to transfer sample holder from station to vacuum chamber with the help of Leica shuttle, D) Vacuum chamber-sample kept in front of pin hole in a cold environment and E) Diffraction pattern obtain using Matlab with every beam hit on sample.

3. RESULTS

3.1 Chromosomes Spreads with Fluorescence Microscope

Chromosomes prepared with above mentioned procedure under materials and methods gave quite good chromosomes spreads as well as single nuclei & chromosomes (shown in figure 6). Though spreads is not clear with 10X but density can be seen, this sample further scan with 63X objective to show clear view of spreads which is shown in figure 7. DAPI stain chromosomes observed under fluorescence microscope gave us good sample, furthermore the morphology and density of the samples was suitable for them to be used for further for various other applications like cryo-imaging.

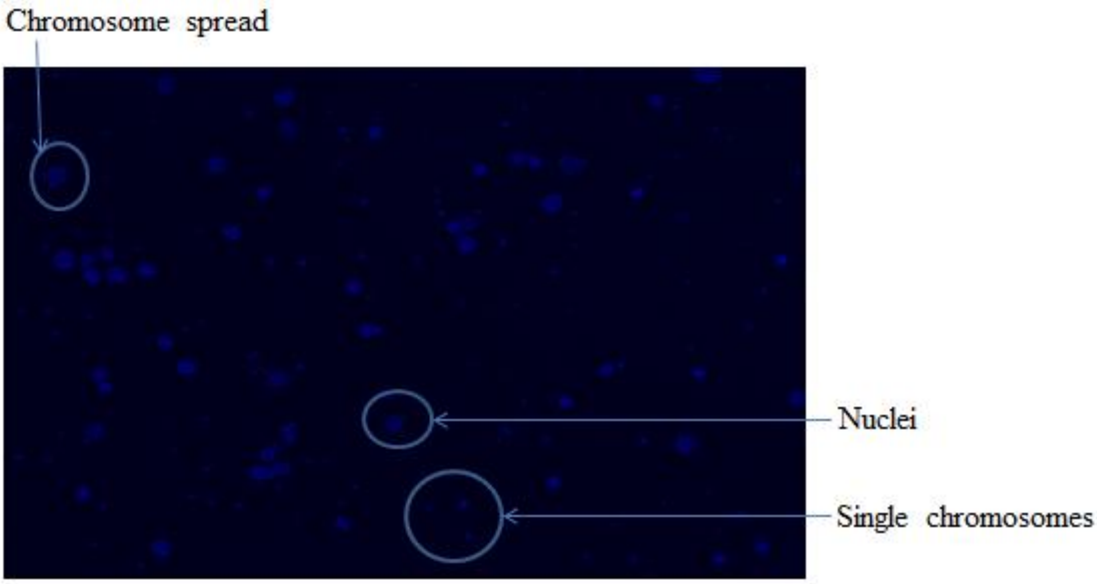

Figure 6: Chromosomes spreads under Fluorescence microscope using objective 10X. Circle indicates spreads, nuclei and single chromosomes.

3.2 Chromosomes Images using the Fluorescence and Confocal Microscope

Methanol acetic acid prepared chromosomes were observed using a confocal and fluorescence microscope for quality and density. Chromosome prepared for confocal were visualised without any stain but for fluorescence imaging, DAPI (a DNA specific stain) was used.

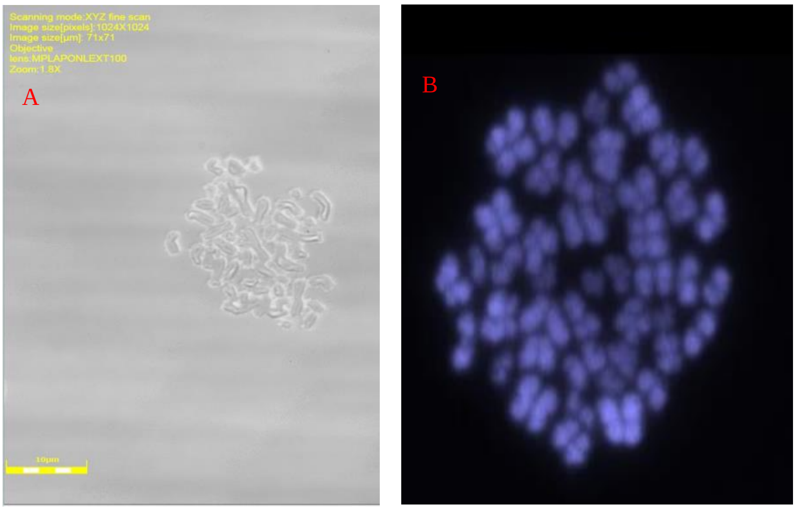

Figure 7: Methanol Acetic acid prepared human chromosomes. 46 chromosomes showed using confocal (A), scale bar-10µm, 100 x objectives and Fluorescence (B) microscope, 63X objectives.

3.3 Transmission Electron Microscopy of Dried Samples

Gold nanoparticle (200 nm) on silicon nitride membranes, were observed under transmission electron microscope as test objects. Chromosomes on silicon nitride windows, stained with uranyl acetate were also observed using the TEM.

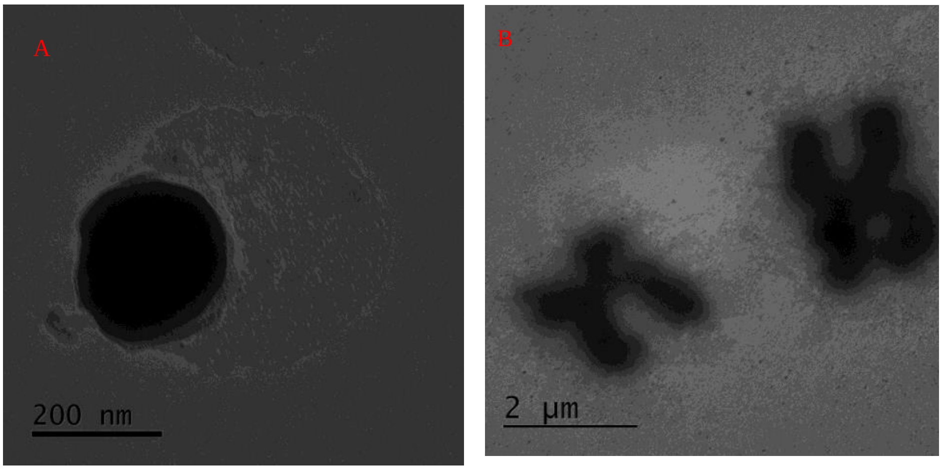

Figure 8: UA stained TEM images A) black circular gold beads, scale bar-200nm and B) Morphology of human chromosomes and scale bar-2µm.

3.4 Recovered Samples

Few grid samples were recovered after x-ray beam scanning. After 8 or 9 scan, samples were taken off from sample holder at room temperature and analysed under confocal microscope to see sample presence or absence on both grids. Black circle shows the presence of sample on both the grid.

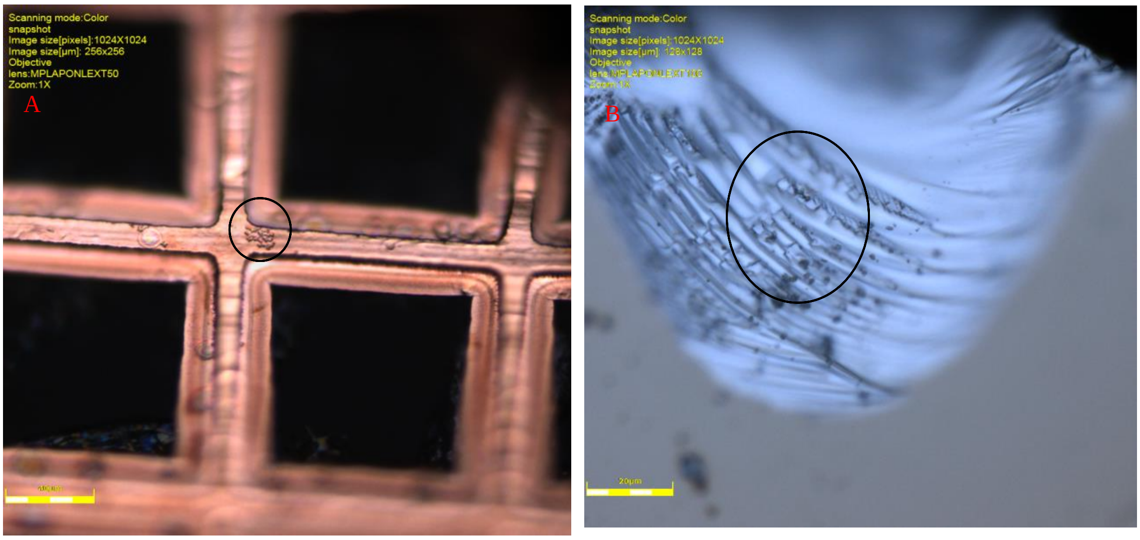

Figure 9: Recovered A) Quantifoil (chromosomes sample on the bar), scale bar-40µm and B) silicon grid (Chromosome sample with Platinum stain on the membrane), scale bar- 20µm, analysed under confocal microscope using 50X objective.

4. Discussions

Suitable human chromosomes were prepared that consisted of 46 chromosomes using both the fluorescence and confocal microscopes. The fluorescence microscope proved to be better as DAPI stain was used and samples could be located easily. The confocal microscope showed chromosomes with no stain but proved to be more problematic to find its morphology. Some chromosome sampled were stained using a DNA specific stain, Platinum blue, as this could provide better contrast during x-ray imaging .

4.1 Hydrophilization of Grids

Two different grids i) silicon nitride (250 x 250 micron window size), 30nm thickness and ii) Carbon-coated quantifoil grids (7 x 7 micron) were selected for spreading the chromosomes on top. It was important to make the surface of the grids hydrophilic as the methanol acetic acid solution (in which the chromosomes were prepared) would not settle on the hydrophobic surface. Successful methods used poly-lysine treatment and glow discharge proved simple; hydrophilization with both the methods does not showed any noticeable change in making grid hydrophilic.

Initial tests to see if chromosomes were settling on the membranes was done by dropping the MAA chromosomes on the grid (silicon nitride) and performing TEM after negative Uranyl Acetate (UA) staining. It is clear that allowing the chromosomes to settle after air drying allows chromosome to be visualized inside the silicon nitride window. Gold beads were done as a test in parallel to the chromosome samples.

4.2 Resistance of Grids

Sample presence on grids was examined by dropping it on the grid which was then observed under a confocal microscope before plunge freezing. It was noticed that inner grid of quantifoil was getting dissolved slowly due to sample drying at room temperature or due to heat from the confocal microscope. This made difficult to see the sample on the grids, but the larger bars of quantifoil were visible. Recovered quantifoil from beamline also had broken grids (Figure 8(A)) which might prove that quantifoil was not of good quality which could not resist the either MAA chemical or was not of good heat resistance.

Silicon nitride window looked undamaged while scanning under confocal microscope; it could resist the MAA-fixative sample at room temperature. When the sample was recovered after X-ray experiment, the silicon membrane was broken that might be due to mishandling while transferring into holder or taking it out at room temperature after several scan.

4.3 Storage of Plunge Frozen Chromosome Samples

Samples that are fixed will not represent the true nature as they would not be in their native state therefore we decided to freeze the chromosome samples. This was done by plunge freezing the chromosomes on the two different types of grids as mentioned earlier. The sample grids were vitrified in liquid ethane to maintain the native state of chromosomes. Once the chromosomes samples were plunge frozen they were under liquid nitrogen stored into grid disc, that were the placed into liquid nitrogen dewar because sample taken out of it can form ice crystal on sample and can interfere in imaging. It would have been useful to image the plunge frozen samples on the Cryo-TEM before however the system was broken and this was not possible.

4.4 Temperamental Stage

The most temperamental part of the sample prep procedure was transferring the sample grid under liquid nitrogen to the sample holder as this involved keeping all components (tweezers, the screw opener) cool in liquid nitrogen. There is the severe risk of ice crystal formation if any mistakes were made. Enough liquid nitrogen should be there in foam dewar to get drowned all components plus able to see through it to operate sample transfer.

4.5 Post Examined Samples

4 samples were recovered at room temperature out of which 2 showed presence of chromosomes i) silicon nitride with platinum stain, ii) quantifoil with MAA- fixative chromosomes ,but two did not show any sample on the grid when observed under confocal microscope and both were platinum stained grids. It was observed that some samples were still present on the bars of (shown in figure 8) the quantifoil but the inner grid was completely broken. A few chromosomes were also seen on the broken membrane of silicon nitride membrane but the morphology of chromosomes was not very clear on grid.

4.6 Future Work

To plunge freeze more quantifoils with same MAA-fixative chromosomes and observe samples using a Cryo-TEM. Recover samples after X-ray experiment using cryo-TEM.

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References

1) A. Estandarte, *A Review of the different Staining Techniques for Human Metaphase Chromosomes,* 2012, University College London.
2) K. Fukui, H. Takata and S. Uchiyama, *Preparation Methods of Human Metaphase Chromosomes for their Proteome Analysis*. Methods Molecular Biology, 2008. 432: p.149-160.
3) M.J.Dobro, L.A. Melanson, G.J. Jensen and A.W. McDowall, *Plunge Freezing for Electron Cryomicroscopy*. Methods Enzymology, 2010.481:p.63-82.
4) L.A.Shemilt, A.K.C, Estandarte, M. Yusuf and I.K. Robinson, Scanning electron microscope studies of human metaphase chromosomes, 2014.