STUDY OF CHROMOSOMES USING PTYCHOGRAPHIC PHASE RETRIEVAL METHOD

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3 November 2013

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Abstract

Ptychographic phase retrieval method was used to reconstruct images of spreads of chromosomes. Different optical set-ups were investigated to increase the resolution of the reconstructions. The associated quantitative phase measurements permitted to determine the volumes of certain chromosomes. These same chromosomes were then imaged using a confocal microscope and their volumes calculated using the obtained measurements of the height. Relative volumes between chromosomes differed for the two imaging methods and did not fully agree with expectations from DNA content. In order to obtain accurate images and volumes of chromosomes the resolution of the ptychographic reconstructions must be increased. Several factors of improvement were discussed in this report.

I-Introduction

Chromosomes have been studied for long using various techniques, but scientists still question some of their basic properties. The method of compaction which allows about 2 metres of double-stranded DNA to be packed into chromosomes is not fully understood [1]. The interest for the volume of chromosomes and its relation to DNA content was particularly important in the early 70's. Different technics such as light microscopy and serial electron microscopy were used to image the chromosomes of higher plant species and determine their volumes [2]. In this experiment ptychography offers a different approach to chromosome imaging and volume determination. Ptychography permits to retrieve a quantitative phase of the object which is lost in classical imaging techniques. This gives information not only on the surface of the object but also on its inside. The change in phase occurring when the light passes through the object depends on its thickness and its refractive index. If the latest is known, the thickness and the volume of chromosomes can be determined by retrieving the phase.

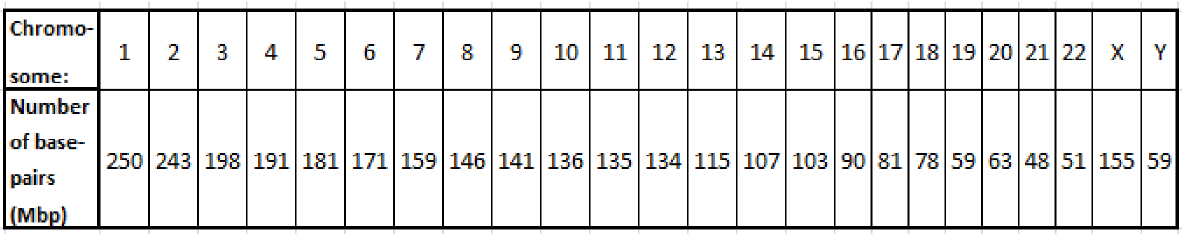
II-Theory

Human genome

The human genome consists of approximately three billion DNA base-pairs carried on 23 different chromosomes. The DNA content of each chromosome[1], found using sequencing methods, is illustrated in Table 1. Various scientists have observed linear relationchip between chromosomes volume and their DNA content using light microscopy. In 1969, Barlow and Vosa observed a correlation coefficient of 0.99 between the volume and DNA content of metaphase chromosomes of the plant *Scilla libanotica* [2]. Is this correlation coefficient the same throughout the cell cycle? How does the volume change throughout the different phases and processes occurring? These questions remain largely unexplored.

Ptychography

Ptychogaphy is a lens-less diffraction imaging techniques which reconstructs the image of an object from its diffraction pattern using a coherent illumination. The phase of the scattered



Chromosome:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
Number of base-pairs (Mbp)	250	243	198	191	181	171	159	146	141	136	135	134	115	107	103	90	81	78	59	63	48	51	155	59

Table 1: Number of DNA base-pairs per chromosomes of the Human genome.

radiation, which is lost in the intensities measurements, is retrieved via iterative algorithms. The object is moved to many overlapping positions to obtain varying sets of information about the object. The iterative algorithm used in this experiment is named Ptychographic Iterative Engine (PIE) and was proposed by Rodenburg and Faulkner in 2004 [3]. It was then improved in 2009 by Maiden and Rodenburg by including an update function for the probe [4].This algorithm permits to retrieve both the probe and the object wave front. The thickness, *t* of the object can be related to its quantitative phase, ϕ by the relation:

$$t = \frac{\phi\lambda}{2\pi(n-1)} \quad , \tag{1}$$

where λ is the wavelength of the incoming radiation and *n* the refractive index of the object.

III-Experimental procedures

Human Chromosomes samples were prepared from b-lymphocyte cells from a Yoruba cell line (GM18507) at passage 4. The cells preparation followed the protocol as described by Jefferson et al, 2010 with slight modifications [5]. They were cultured in suspension in RPMI-1640 medium supplemented with 20% foetal bovine serum and 1% L-Glutamine at 37°C in a 5% CO_2 incubator. Colcemid (Invitrogen, UK) at a final concentration of 0.2 µg mL^{-1} was added to obtain metaphase chromosomes. After six hours, the cells were centrifuged, resuspended in pre-warmed hypotonic solution (0.075 M potassium chloride) for 5 minutes and fixed in three changes of 3:1 methanol. Chromosomes were placed on a slide using a dropping method to optimise the formation of spreads. The sample was stained with DAPI to control the amount and the quality of spreads with fluorescent microscopy.

The slides were inserted in the set up illustrated in figure 1. A diode laser of 406nm wavelength was used as source of coherent light. The laser passed through a condensing lens, which insures a maximum of the light to arrive to a pinhole. The slide was placed as close as

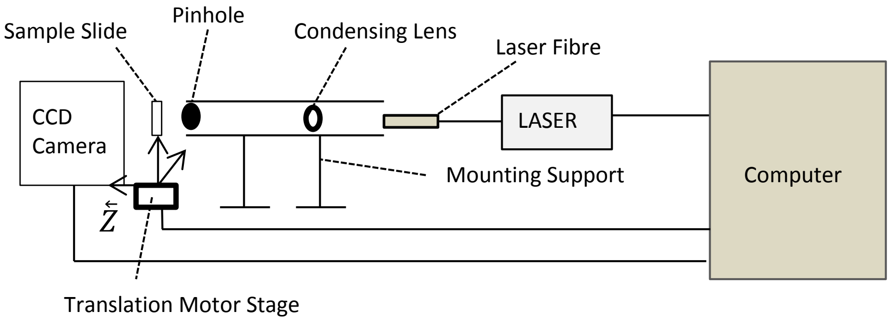


Figure1: Diagram of the experimental set-up 1.

possible from the pinhole. The sample holder was linked to a translation motor stage to accurately control the position of the slide. It could be moved in three orthogonal directions: the optical axis was set as the Z-axis, indicated on Figure 1. The diffracted light was detected by a sCMOS CCD camera. The diffraction pattern recorded could then be used to reconstruct an image of the sample using the algorithm PIE. The alignment of the translation plan and the imaging plan of the CCD camera was done manually by comparing distances between the front of CCD camera and the slide while moving the stage in one direction. This alignment was necessary, as a change in distance from the object to the detector between two points during scanning will affect the wave propagation performed during the reconstruction.

The resolution being the main concern, the technique was first tested using a “strong” sample of flower seeds. The size and the high contrast between the features of the object would permit an easier reconstruction. Different optical set-ups were experimented. The first simpler case, illustrated in Figure 1, contained only a pinhole (set-up 1). In the second set up a diffuser of one degree was added before the pinhole (set-up 2). This increased the numerical aperture and, therefore increased the resolution. In the third set up (set-up 3), a lens replaced the pinhole to ensure all the light to hit the sample and to increase as well the numerical aperture. The setting which was found to increase the resolution the most was the combination of a diffuser and a pinhole. The pinhole was chosen small: about 0.5mm. A small pinhole meant a small illuminated region and therefore a small region scanned per position. This should increase the quality of the reconstruction as more information per area scanned is recorded. The position of the diffuser with respect to the pinhole was varied along the optical axis and was set to about 4cm. More studies on the effect of the diffuser could be undertaken to get the position with the best increase in resolution. Another concern was the strength of the diffuser which could possibly affect the reconstruction. If too strong, small complicated speckles would be observed, giving a complicated probe to reconstruct.

Step	**Imaged Object**	**Set-up**	**Chip Size (pixels)**	**Best Known Function**	**Initial Probe Guess**	**Initial Object Guess**
1	Tissue	1	1024	Probe	Pinhole model	none
2	Spread	1	1024	Probe	Probe reconstruction from step 1	none
3	Tissue	2	1024	Object	Probe reconstruction from step 1	Tissue reconstruction from step 1
4	Spread	2	1024	Probe	Probe reconstruction from step 3	Spread reconstruction from step 2
5	Tissue	2	2048	Object	none	Interpolation of tissue reconstruction from step 3
6	Spread	2	2048	Probe	Probe reconstruction from step 5	Interpolation of spread reconstruction from step 4

Table 2: Main steps of the imaging and reconstruction method. The set-up (1:pinhole or 2:pinhole + diffuser) and the size of the CCD camera chip used for imaging are indicated. The method consisted of achieving a good quality of the initial guess of the object for one particular set-up. Then, this reconstruction was used to retrieve a probe which was different or more complicated, due to the addition of diffuser or the change of the CCD camera chip size.

Scans were performed over a 1× 1mm region with a step size of 0.1mm. As expected, the chromosomes were too small and “weak” to reconstruct an image directly. The large amount of empty space in the sample from which almost none information could be extracted was making convergence to a solution difficult, giving a bad quality of reconstruction. A piece of tissue was stick to the slide close to the chromosomes spread. It was used as a strong sample to reconstruct an accurate probe. A trace of pen was also investigated as a strong sample, but it was difficult to discern the correct focus plan as no small features were present. The reconstruction method is indicated in table 2. Its aim was to obtain, by imaging a strong sample, a reconstruction of the probe accurate enough to retrieve a good reconstruction of the chromosomes spread. Because of the height difference between the two objects this could not be fully achieved, as explained in more detail in the next section. For the step 6, in table

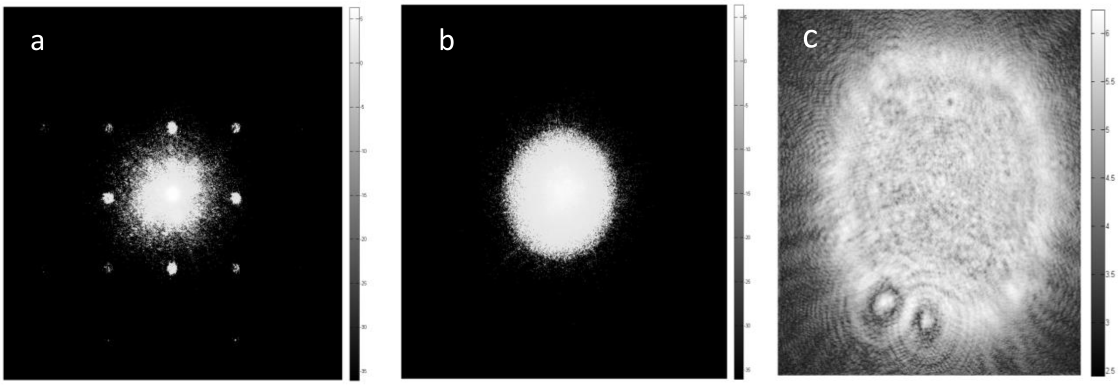


Figure 2: a) diffraction pattern recorded when imaging a chromosome spread using set-up 1; b) diffraction pattern recorded when imaging a chromosome spread using set-up 2; c) diffraction pattern recorded when imaging the strong sample of flower seeds using set-up 3. In a) and b) the full size of the camera chip was used (2048×2048 pixels). In c) half of the chip size was used for measurements (1024×1024 *pixels).*

2, better results were observed when doing first a reconstruction for which the interpolation of the object was the best known function. The probe was updated during the reconstruction. Hence, further reconstructions were performed using the probe as best known function. After imaging three different spreads using ptychography, the chromosomes sample was imaged using a confocal microscope. An M FISH was then performed to identify the chromosomes present in one of the imaged spread.

IV-Results and Discussion

Reconstruction and Imaging

The diffraction patterns obtained using the three different set- ups are shown in figure 2. By comparing figure 2.a and 2.b, which were taking using set-ups 1 and 2 respectively, the increase in numerical aperture due to the addition of the diffuser can be observed. The pixels of the CCD camera screen acted as a diffraction grating. The different diffracted orders of the pinhole, which can clearly be observed in figure 2.a, were reflected back to the camera by the slide. In figure 2.b, these reflections were not observed due to the action of the diffuser. In the third set up, some dusts of similar size as small features of the strong sample were observed on the lens. This could explain why no improvements in resolution or quality were observed. Another possibility would be the loss of information due to the use of half-size CCD camera chip. No further measurements were taken as the lens was not clean but the use of this set up

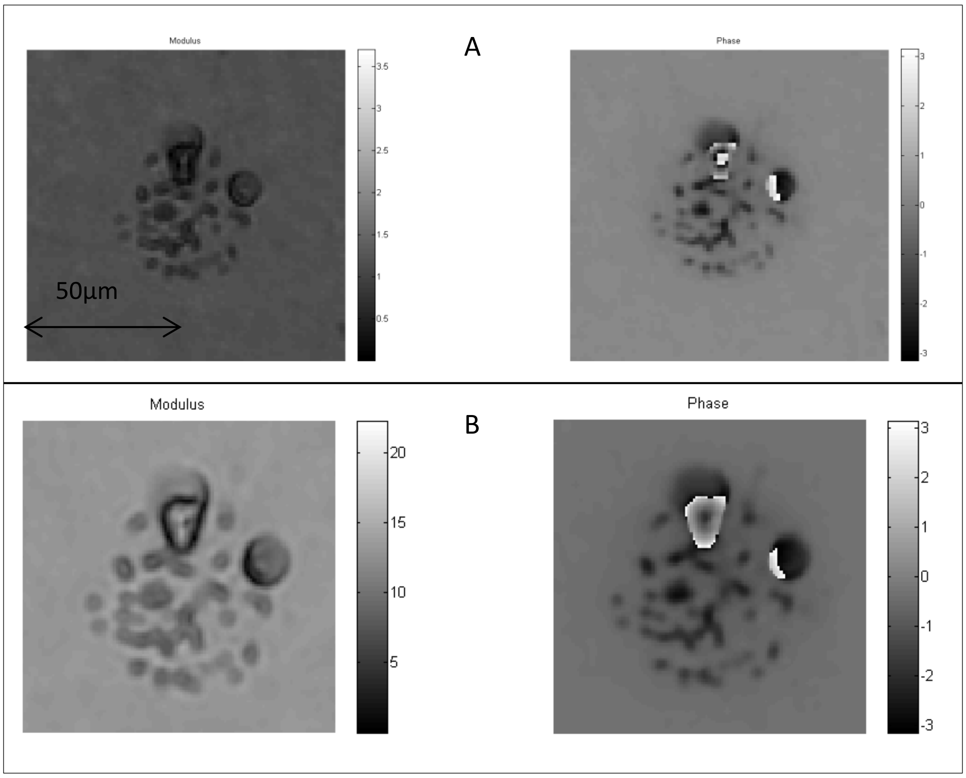


Figure 3: A) is the modulus (left) and phase (right) reconstructions of the first spread of chromosomes[6]. The diffraction patterns were recorded using set-up 1 and half the size of the camera chip. B) Shows the reconstructions of the same spread when the diffuser was added to the set-up and the full size of the camera chip used.

should be investigated in future experiments. Three different spreads of chromosomes were imaged. Comparing figure 3.A and 3.B, which shows the reconstruction of the first spread, the increase in resolution from the addition of the diffuser can be observed. The strong sample used to obtain these images was a piece of tissue. Its modulus and phase reconstructions were not net. This was certainly due to the complexity of the object and to its thickness. It is important in ptychography to use a sample which is extremely thin. This reconstruction method assumes the object to be two dimensional. Therefore some averaging between possible solutions or focus planes will be performed if the object is thick, leading to a decrease of the quality of the reconstruction. This thickness problem could be observed in images of the third chromosomes spread, as seen in figure 4. A pen trace was made on the other side of the slide. Information was received from two objects separated by about 1mm. It could be seen that the quality of the reconstruction decreased when approaching the pen trace.   For future experiment it is primordial to find a ‘strong’ sample of the same thickness

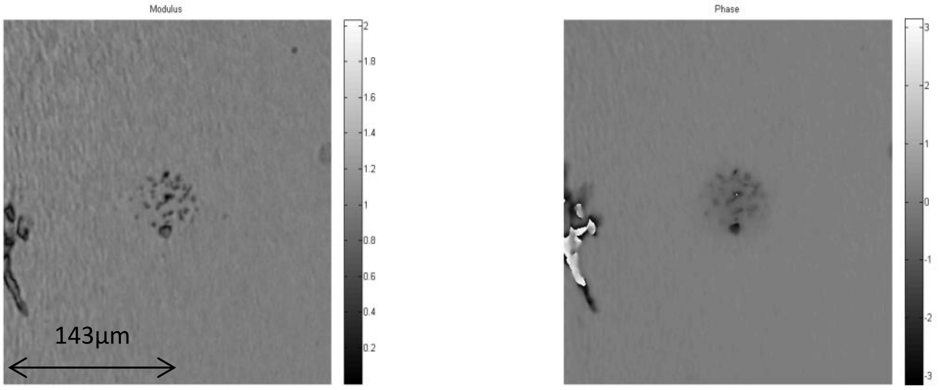


Figure 4: The modulus (left) and the phase (right) reconstructions of the third spread of chromosomes [7]. The diffraction patterns were recorded using set-up 2 and the full size of the camera chip.

as chromosomes. A small difference in distance from the camera to the object will affect the wave propagation performed to reconstruct the image. Even if the quality of the strong sample is good, it must be in the same plane as the chromosomes sample in order to provide an accurate starting probe guess. The probe reconstruction associated with the object reconstruction of figure 3.B, and the reconstructions of the second spread are shown in the appendix A.

An image of the first spread of chromosomes taken using a confocal microscope can be seen in figure 5. The expected metaphase X-shape of chromosomes can clearly be observed. An M-FISH technique was performed to identify the chromosomes of the first spread imaged with ptychography and the confocal microscope, as seen in figure 6. It was successful except for a few chromosomes for which the colour did not match with expectations. This could be due to the proximity between chromosomes or small damages due to their exposition to the laser.

Volume analysis

The volumes of certain chromosomes were calculated using both data sets from ptychography and from confocal microscopy. In the two cases, height measurements were integrated over a chosen area. From ptychography data, the height or thickness was calculated from the retrieved phase and a value of the refractive index of DNA thin films (n=1.54), found in literature[11]. The confocal microscope performed a three dimensional scan. Height measurements were directly obtained. The integration of the height measurements was first performed using a tool of the imaging software of the confocal microscope, and then, for

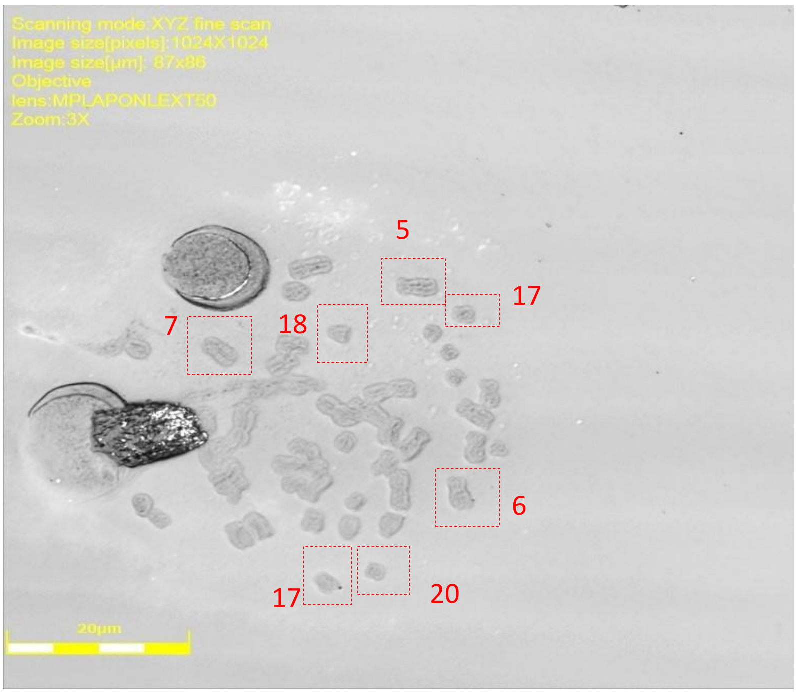


Figure 5: Image of the first spread of chromosomes obtained with a confocal microscope [8]*. Chromosomes for which the volume was determined and the "genome number" to which they were identified are indicated.*

comparison, performed directly from the data using numerical integration. Results of the three methods are shown in table 3. In the three cases, the volumes calculations depended on the threshold chosen. A difference in height between the direct surrounding of chromosomes and a flat area far from the spread was observed. While using the implemented volumes calculation tool of the confocal microscope software, the background value was set to be the one of the direct surrounding of chromosomes. This technique gave a much smaller value than if the background was set to be the value of the flattest region of the scan, as done for the two other calculations methods. The difficulty was to dissociate the material surrounding chromosomes from the background and from the chromosomes itself. With the implemented software tool, the area selection and integration were accurate, but the threshold defined the background and not only the selected area. If we assume that some material such as the nucleoplasm was present next to the chromosome, then some part of the volume of the chromosome was lost when setting the threshold. For calculations realized directly from the data of the confocal microscope, the threshold was not used, and only a background subtraction was performed. But the area selection must be improved as the background was not the same everywhere. For example, one of the chromosomes was found between two nuclei. The surrounding background was much more important than for the other

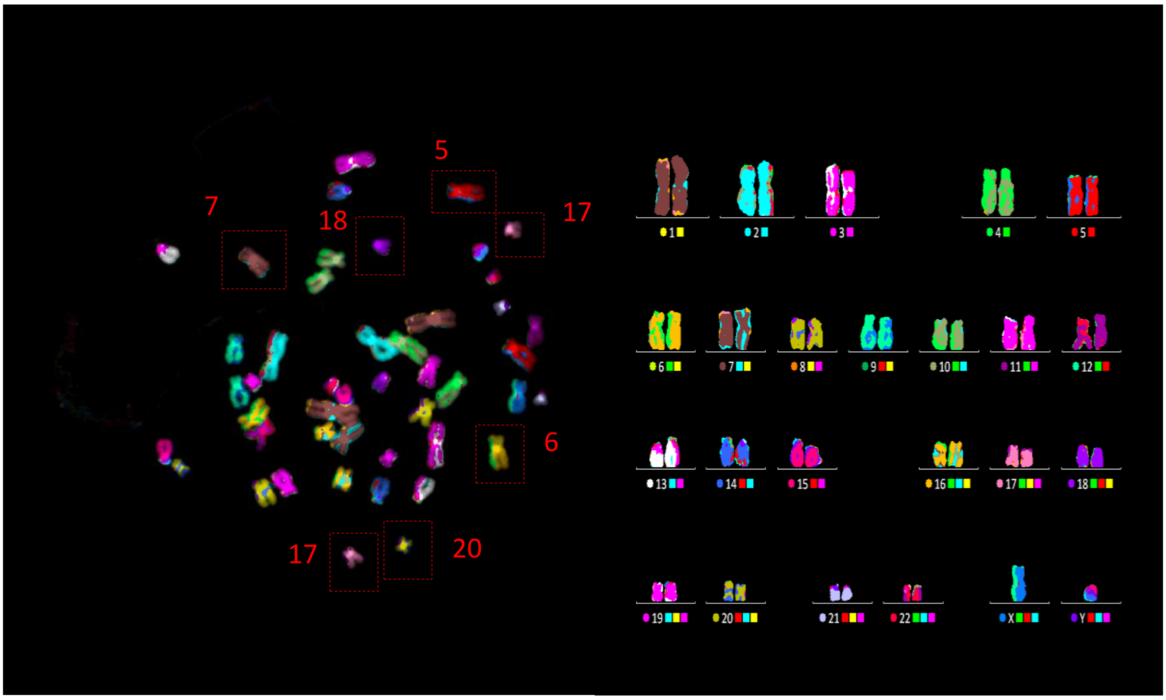


Figure 6: M-FISH of the first spread of chromosomes [9]*. Chromosomes for which the volume was determined and the "genome number" to which they were identified are indicated.*

chromosomes, affecting the calculation. For calculations from ptychography measurements, the threshold was used only to select the area over which the height integration was performed. The background subtraction was done independently. As each chromosome reconstruction was composed of a few pixels, the area selection could not be accurate and an additional pixel was changing the results considerably. As seen in table 3, results were of the same order. The relative volumes do not fully agree with the expectation from DNA content. With this resolution and accuracy, these techniques could not be used to differentiate chromosomes of the same size. It would require a much better resolution to be able to sort chromosomes out accurately using ptychography.

Few factors affecting the resolution should be investigated in future experiments. One of them, as previously stated, would be the three dimensional character of the strong sample which affects the accuracy of starting probe guess. Another factor could be the laser instability. The oscillation of the intensity of the laser source during the scanning and its effect on the reconstructions should be studied. The lack of information obtained from the empty spaces surrounding the chromosomes spreads, which makes convergence to a solution difficult, could be solved by increasing the density of the sample. Other suggestions to

	DNA content (M base-pair)	Volume from Ptychography (μm^3)	Volume from Confocal 1 (μm^3)	Volume from Confocal 2 (μm^3)
Chromosome 5	181	2.11 ± 0.09	0.90 ± 0.2	3.6 ± 0.4
Chromosome 6	171	1.8 ± 0.1	1.1 ± 0.2	4.1 ± 0.4
Chromosome 7	159	2.5 ± 0.4	1.1 ± 0.2	4.5 ± 0.5
Chromosome 17	81	1.19 ± 0.06	0.36 ± 0.08	1.9 ± 0.3
Chromosome 17	81	0.8 ± 0.1	0.42 ± 0.09	1.9 ± 0.2
Chromosome 18	78	1.3 ± 0.5	0.43 ± 0.09	2.0 ± 0.3
Chromosome 20	63	0.94 ± 0.03	0.31 ± 0.08	1.6 ± 0.2

Table 3: Volumes of certain chromosomes of the first spread of chromosomes determined from data obtained using ptychography and confocal microscopy. Confocal 1 is the method of volume determination using the implemented tool of the confocal microscope software. Confocal 2 stands for the numerical integration performed directly from the data of the confocal microscope. The corresponding DNA content is indicated for comparison.

improve reconstructions of ptychography could be the use of a smaller pinhole, a better fixation of the set-up and a precise verticality of the slide to avoid phase ramp. In addition to these, an automate way of adding elements such as a diffuser to the main set-up would reduce the error in the alignment of the laser with the centre of the camera chip. Finally, the value of refractive index of chromosomes should be further investigated.

V-Conclusion

Images of three different spreads of chromosomes were reconstructed using ptychography. The volumes of certain chromosomes of the first spread were determined from the phase measurements. For comparison, the first spread was imaged using confocal and fluorescence microscopy. Volumes of the same chromosomes were calculated from the height measurements obtained with the confocal microscope. Results were of the same order but relative volumes were different for the two imaging methods, and did not fully agree with expectations from DNA content. One of the reasons for these differences was the dependence of the volume on the threshold or on the surrounding of chromosomes. In addition to this, the low resolution of ptychography limited considerably the accuracy of the calculations. In order for ptychography to be a useful methods to image chromosomes and determine their volumes, higher resolution must be achieved. Beyond to the limitation from the optical geometry of the set-up, certain factor such as the laser instability could lower the resolution and should be further investigated.

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Appendix A

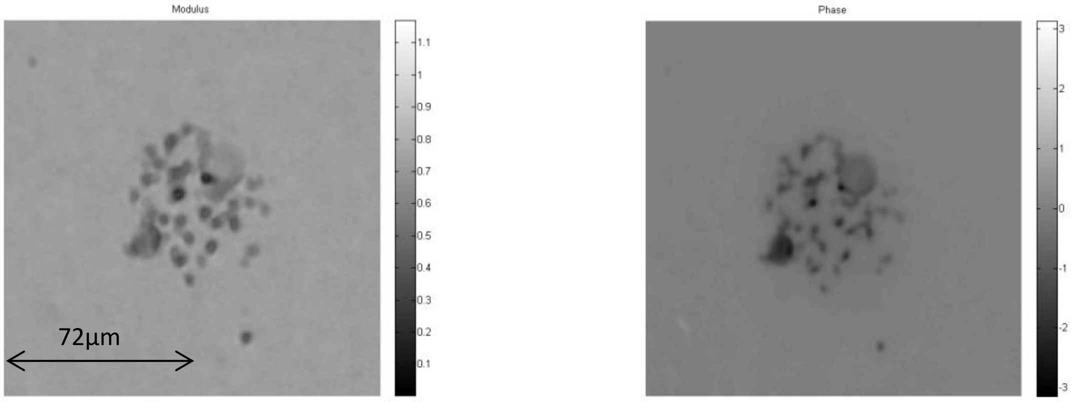

Figure 7: The modulus (left) and the phase (right) reconstructions of the second spread of chromosomes[10]*. The diffraction patterns were recorded using set-up 2 and the full size of the camera chip.*

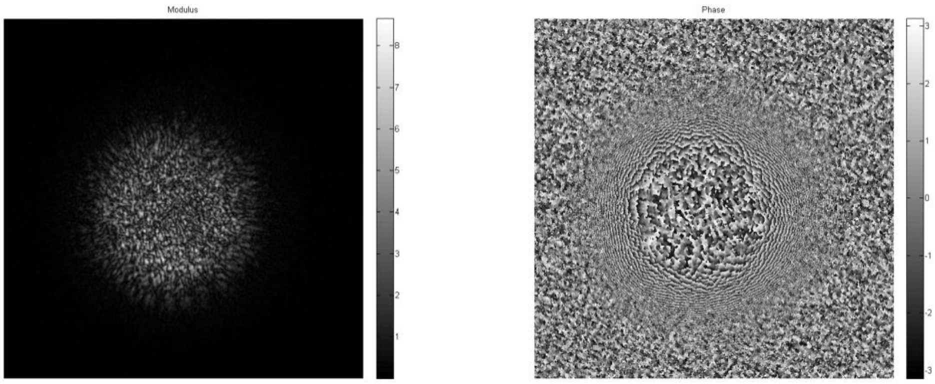

Figure 8: The modulus (left) and the phase (right) reconstructions of the probe[6] *used to image the first spread of chromosomes shown in Figure 3. The diffraction patterns were recorded using set-up 2 and the full size of the camera chip (2048 × 2048 pixels)*

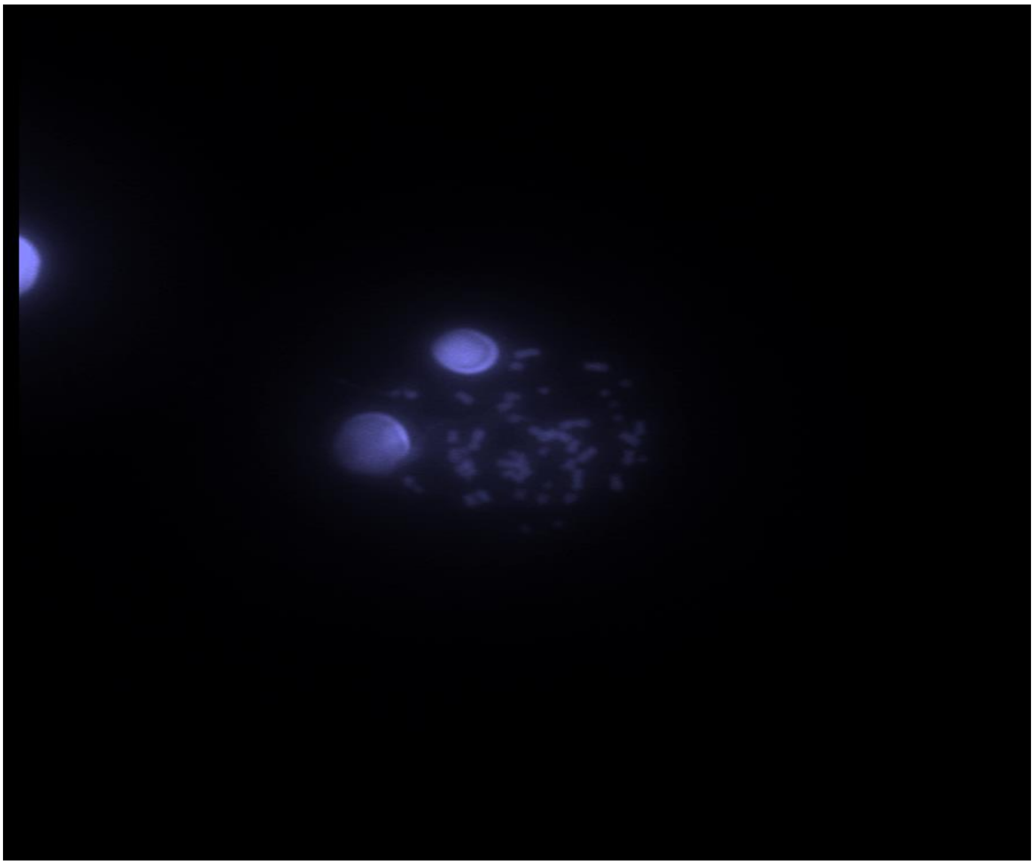

Figure 9: First spread of chromosomes imaged with a Zeiss AxioZ2 microscope using DAPI illumination.[9]