An Accurate Image Processing Algorithm for Detecting FISH Probe Locations Relative to Chromosome Landmarks on DAPI Stained Metaphase Chromosome Images

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Abstract

With the increasing use of Fluorescence In Situ Hybridization (FISH) probes as markers for certain genetic sequences, the requirement of a proper image processing framework is becoming a necessity to accurately detect these probe signal locations in relation to the centerline of the chromosome. Although many image processing techniques have been developed for chromosomal analysis, they fail to provide reliable results in segmenting and extracting the centerline of chromosomes due to the high variability in shape of chromosomes on microscope slides. In this paper we propose a hybrid algorithm that utilizes Gradient Vector Flow active contours, Discrete Curve Evolution based skeleton pruning and morphological thinning to provide a robust and accurate centerline of the chromosome, which is then used for the measurement of the FISH probe signals. The ability to accurately detect FISH probe locations with respective to the centerline and other landmarks can provide the cytogeneticists with detailed information that could lead to a faster diagnosis.

1. Introduction

Many chronic diseases can be traced back to the DNA structure of a patient. Therefore the study of human chromosomes and their structure becomes of utmost importance in clinical diagnosis. Non radioactive Fluorescence In Situ Hybridization (FISH) has been used to assist this diagnosis process by providing the cytogeneticist with information regarding the present location of a known DNA sequence in a selected chromosome, which could be used to detect certain chromosomal abnormalities [18]. FISH uses fluorescence DNA probes to detect chromosome sequence rearrangements in genetic diseases. Karyotype analysis is one of the main research areas in image processing which aims at producing annotated karyograms with the least user involvement. Methods available for karyotyping or other chromosome analysis are mainly limited by the shape variability caused by non-rigid nature of the chromosome structure. Therefore, the effectiveness of these image processing techniques are highly limited by the inability to provide proper results irrespective of the shape of the chromosome [19]. Proper segmentation and extraction of the center line of the chromosome plays a vital role in many of the available karyotype analysis methods [8],[21]. In this research, the image processing techniques are applied to DAPI (4',6-Diamidino-2-Phenylindole) stained chromosome images in contrast to Geimsa stained images used in many karyotype analysis methods in literature.

Karyotype analysis is used in this paper merely for comparison of methodologies used for information extraction and our algorithm can be readily adopted in to any type of analysis which needs similar information. In this research, we have identified the following features as the most important landmarks for FISH probe projection [5],[20],[21],

- A proper segmentation of the chromosome.
- An accurate centerline extraction.
- Telomere coordinates and the centromere location.

In general, segmentation methodologies applied to chromosome images vary from very simple to highly complex in computational aspects. The common method used is global or local thresholding [4],[5],[24]. This simple method gives good results in chromosome images due to the fact that the images only have 2 modes (object/background) and binarization usually yields distinct chromosome boundaries. Yet, thresholding tends to be sensitive to noise and image in homogeneities as spatial information is not considered in the decision making process. Another segmentation method found in literature is parametric deformable mod-

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els. Among these, Gradient Vector Flow (GVF) based active contours have been proven to deliver better results, especially in chromosome image segmentation [2],[13]. First introduced into active contours in 1997, GVF snakes addressed a main limitation in the traditional active contours [25] by drastically improving its capture range.

Medial Axis Transform (MAT) and morphological thinning are the most commonly adopted methods in finding the centerline of chromosomes, but they suffer from many inherent limitations. MAT provides a set of points in space, rather than a parametric curve that can be effectively and easily used for further calculations. Several attempts have been made in order to find suitable methods without using skeletonization or thinning. Jim Piper and Erick Granum [19] proposed a two stage approach to find the centerline in which they first determined the orientation of the chromosome by calculating the minimum width enclosing rectangle. Then, if the chromosome is not highly bent, it was rotated such that the orientation is vertical and mid points of the horizontal chromosome slices were connected together to obtain the centerline which was then smoothed to get the "poor man's skeleton". But, if the chromosome is bent, they performed a conventional skeletonization algorithm. Yet, the problem with this approach is the spurious branches that occurred with the conventional skeletonization process. In another approach [8], chromosomes were sampled into scan lines of different inclinations and after selecting proper cross-sections, the selected mid points were combined to obtain an approximate centerline. The drawback of this method is that it attempted at getting a polygonal approximation of the centerline instead of the centerline itself. Results were poor when the segmented chromosome boundaries were irregular in shape, which is a common occurrence in medial imaging. Gunter Ritter [21] proposed a method which was based on finding the dominant points of the chromosome. But, results were not reliable when it was applied to highly bent and blurred chromosomes.

Apart from the methods mentioned above, the majority of chromosome centerline findings are carried out with the following two steps,

1. Iterative morphological thinning or skeletonization process which reduces the 2D binary image to a collection of points representing the original shape information of the object.

2. A Pruning process which is used to remove all the spurious branches present in the skeleton obtained above.

There have been many approaches in the field of karyotyping, where the centromere location information was used to classify chromosomes into different classes [15],[16],[22]. Some of the centromere detecting work was based on methods that did not involve finding the centerline of the chromosome. Mousavi [23] assigned a membership value for each pixel of DAPI and FITC images (with centromere probes) based on an iterative fuzzy algorithm. Another work carried out by Moradi [16] and similarly by Faria [3] (on fish chromosomes) attempted to find the centromere location by getting the horizontal and vertical projection vectors of the binary segmented chromosomes. Most of these methods did not perform well on acrocentric chromosomes as well as on chromosomes with a bend greater than 90^0 degrees. Majority of work carried out on centromere location is based on getting the centerline of the chromosome first. Piper's [19] approach towards this was to achieve the second moment of the profile of the chromosome along its centerline. Moradi [15] took the average of image intensities along scan lines perpendicular to the centerline and used wavelet de-noising to remove sharp perturbation in the density profile (DP). Wang [22] extracted the shape profile, density profile and the banding patterns using scan line sampling and then used a rule based setup to detect the chromosome centromere.

The proposed hybrid algorithm which is based on GVF snakes, DCE based pruning and morphological thinning is explained in detail in the next section 2.

2. Proposed algorithm

At the outset, the fluorescence microscopy images obtained from a single specimen were subjected to a ranking algorithm[10]. This algorithm provided a ranked set of metaphase images in which chromosome images that are spread well and are complete were ranked higher. The rest of the proposed algorithm deals with the set of images with the best rank so that the accuracy of the overall process is improved.

2.1. Pre processing and segmentation

The initial processing stages prior to the secondary segmentation are listed below,

- Step1: The fluorescent chromosomal images were first normalized and then subjected to global thresholding based on Otsu's method [17]. This thresholding algorithm attempts to segment the histogram of the image into two clusters by minimizing intra-class variance.

- Step2: The contour (boundary pixels) of the above segmentation result was extracted by removing all 4-connected pixels of the binary image.

- Step3: The inverted DAPI chromosome image was further subjected to a 2D median filtering stage to remove any noise and artifacts present in the image while preserving chromosome boundary information.

The rationale for adopting a parametric active contour model was due to the availability of a close approximation of the chromosome shape through thresholding and the presence of strong edges around the chromosomes. Yet, due to unequal illumination across the fields of view, these initial object shapes can be either under-approximated or over-approximated. Therefore, the snake model in question had to be able to either expand or contract into the chromosome object depending on the initial contour positioning. Also, due to the shape variability of chromosomes, we required a snake model that could converge into concave boundaries.

GVF based snakes [25] successfully address all of the above issues and have a significantly higher convergence and capture range compared to the traditional snake model. GVF snakes also have the ability to shrink or expand depending on the gradient vector field which is a diffused field based on the edge map of the image. Unlike Gaussian smoothing which is commonly used to increase the capture range, the use of GVF doesn't blur the edge map and thus leads to more accurate boundary positioning at convergence. GVF based snakes have been used in many segmentation algorithms including human chromosome segmentation [2],[13] and yield satisfactory results compared to other segmentation methods. Therefore, the contour extracted from the reduced binary image was parameterized and those control points were allowed to move into the object boundaries by iterating until convergence.

2.2. Finding the centerline

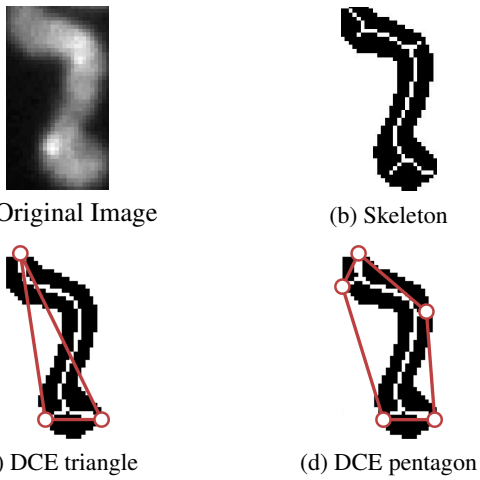
The chromosome centerline is necessary in many operations like classification performed on segmented chromosomes [19],[20]. Many shape and structure-related features such as the chromosomal banding pattern, width and density profiles can be extracted using the centerline. Small deviations in the extraction of these authentic features could result in classification errors [8]. The majority of centerline extracting methods reported in the literature are based on MAT (Medial Axis Transform) and different thinning methodologies [15]. Skeletonization or thinning produces spurious branches frequently at bend locations in particularly towards the telomere regions of the chromosomes. The methods that are not based on MAT mainly have problems with handling objects with sharp bends which are commonly present in metaphase chromosomes [8],[19].

We have adopted a skeleton pruning method based on Discrete Curve Evolution (DCE) [1] which in our algorithm was applied only to chromosomes with skeletons longer than a particular length (35 skeletal points) and shorter chromosomes were processed using the thinning algorithm described by Lam [11]. The rationale behind this hybrid application is to use DCE based pruning only on chromosomes which are highly likely to be bent while utilizing thinning on relatively shorter chromosomes for which the skeleton deviates from the centerline. The DCE based skeleton pruning process is based on partitioning the object contour into polygonal sections and then evolving them according to DCE. Furthermore, pruning is achieved by removing all skeletal points of which all the generating points (the points where the maximal disks touch the object boundary) lie on the same polygon partition. Results in this skeletal pruning method tend to be highly dependent on the contour partitioning itself. Therefore the skeleton pruning problem can be viewed as a contour partitioning problem. DCE provides an ideal solution for this by effectively evolving polygon partitions by vertex deletion based on any given relevance measurement [12]. For the implementation, any digital image boundary can be approximated to a polygon without a loss of information by taking each boundary pixel as a vertex on the polygon and similarly considering the distance between each pixel as an edge. DCE was used to evolve the polygon iteratively by removing the vertex which has the least value for the relevance value $K(v,u,w)$ defined in equation 1, where d_{uv} & d_{vw} are the Euclidian length between the vertices and θ is the turn angle at vertex v. This relevance function was selected so that it is dependent on features of its neighbors and thus makes DCE able to evolve using global features of the shape information.

$$K(v,u,w) = (\theta * d_{uv} * d_{vw})/(d_{uv} + d_{vw}) \qquad (1)$$

Also, as DCE is simply deleting vertices of the polygon partitions, the topology information is guaranteed to be represented at the skeleton ends. Furthermore we have considered only the convex polygon combinations in order to prune spurious branches effectively [1],[12]. Figure 1 depicts the reliability and accuracy of the DCE based pruning method compared to standard pruning. Figure 1(b) and figure 1(c) depict two DCE based pruning results for different number of vertices for the end convex polygon

In the case of obtaining the medial axis of a chromosome, the ideal result would be a pruned skeleton with no extra branches. Yet, as the minimum convex polygon being a triangle and DCE being modeled as polygons, the resulting skeleton will at minimum have one spurious branch. This issue was resolved by tracing all branches and pruning the shortest branch completely. The DCE result was then processed by a modified thinning algorithm to ensure single pixel thickness of the skeleton. Our modified thinning algorithm consisted of the application of a set of masks to the skeleton on the basis of the morphological hit & miss algorithm followed by a thinning process described by Lam [11]. A curve fitting step was then introduced to obtain a smooth curve from the skeleton of the previous step. This step was based on cubic spline interpolation which attempts to fit a 3^{rd} order polynomial between each of its



(a) Original Image (b) Skeleton

(c) DCE triangle (d) DCE pentagon

Figure 1. Comparison between standard skeleton with DCE based solutions.

control points (knots) while keeping continuity at its end point connections. Therefore cubic spline interpolation is an appropriate method for approximating the centerline of any bent metaphase chromosome. The control points for curve fitting were provided by sampling the skeleton result obtained through DCE pruning step and registering a control point for approximately every 7 skeleton points after excluding some portion of each end. The interval of '7' points above was selected empirically in order to avoid over fitting the data while representing the shape information adequately. The end section clipping was performed to remove the skeletal portion that deviates at the telomere regions from the actual centerline.

Table 1. A 7×7 representation of the original 20×20 template used for end point correction, where the coefficients were set as '0' as ignored, '+' as +1 and '-' as -1.

0	0	0	0	0	0	0
0	0	-	-	-	0	0
0	0	-	-	-	0	0
0	-	-	+	-	-	0
0	-	+	+	+	-	0
-	+	+	0	+	+	-
0	+	0	0	0	+	0

Finally, a methodology was developed to correct the end points of the centerline using a heuristic gradient based end point detection method. This was achieved by creating a template which matches telomere regions of a chromosome (see table 1). The line segments used in this were extended segments of the end points of the sampling points used for spline curve fitting stage. The lengths of these segments were selected to be 20% of the centerline.

2.3. Centromere identification

The centromere is the most condensed and constricted region of a chromosome to which the spindle fiber is attached during mitosis (cell division) [9]. The detection of centromere is an important stage in almost all karyotype classification methods as the centromere index value is a key feature which can be used to relate a given chromosome to its group. The centromere index is the ratio between the short arm to the total length of a chromosome, which corresponds to the location of the centromere.

All the earlier mentioned approaches in detecting the centromere are mainly limited by the lack of knowledge of the information relevant to chromosomes as these are merely a section of a karyotype analysis problem. Our application differed from karyotype analysis as the system was meant to analyze a known chromosome at any given instance. Therefore, the centromere identification process can be adapted to include that information to assist the detection process. For an example, the information whether a given chromosome is acrocentric or not would help a great deal for the program to find the centromere. In our approach, the centerline prior to the stage of end point correction was used for this purpose and line segments were sampled perpendicular to that of the centerline segment at unit length intervals. The sampling of the intensities along these perpendicular line segment(referred to as 'trellis' in subsequent sections), was weighted based on a Gaussian function. This was intended to cancel image and boundary noises as well as effects introduced by bending of the chromosomes. Though the sampling of intensities along the trellis was performed on the filtered DAPI image, the length of the trellis segments were decided from the binary result obtained through GVF in section 2.1. The motive behind using the GVF result was to base the trellis on a binary image which had more edge characteristics than a simple thresholded binary image. With this selection, the constriction at the centromere became more pronounced. In our approach, we relied on the following parameters to locate the centromere,

1. The width profile of the chromosome along the trellis on the centerline which was obtained using the GVF binary image result.

2. Density Profile (DP) obtained by getting the weighted average of intensity values of the DAPI image (based on Gaussian function) along the trellis, which is limited by the GVF result.

The challenge in finding the centromere by using the above two factors was in developing a suitable framework

for combining these two data features. We implemented a methodology based on the concepts of 'model based segmentation' in which the features were combined using a prior model designed to bias the most suitable feature over the other feature for each chromosome. Metacentric chromosomes are known to have a clear and more pronounced centromere whereas acrocentric chromosomes are not. In this work we had prior knowledge of the chromosome which we were dealing with. Thus, based on the identity of the chromosome, probability values or biasing factors were calculated using the biasing prior by which the two normalized feature sets were appropriately combined into a single feature set. Centromere location was then obtained by finding the global minimum in this resulting feature set.

2.4. Probe signal projection

Our objective as stated in section 1, is to detect the location of any number of FISH probes with respect to the main chromosome landmarks, namely the locations of the centromere and the telomere. Therefore, an accurate projection of the probe signal onto the measuring grid (which is the centerline in this case), is important. Many iterative point projection methods were developed over the years which are of zero, first and second order [6],[7],[14]. The order of the projection methodology mainly relies on the degree of variation of the test point (to be projected) with that of the target surface or curve. Therefore, a simple first order tangent based orthogonal projection method was selected for this purpose and it is depicted by Figure 2.

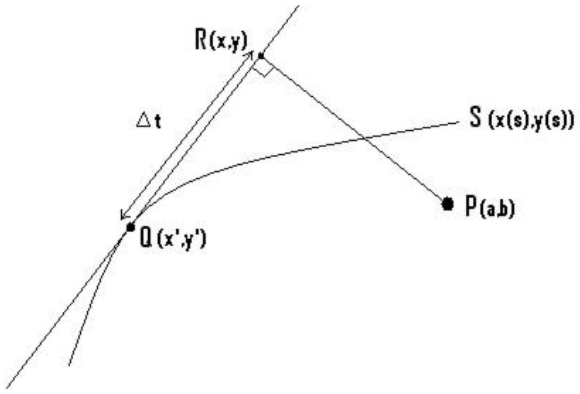


Figure 2. The tangent based method setup.

In the setup in Figure 2, 'P' is the point that needs to be projected (with known coordinates (a, b)) onto the curve 'S' which is a parameterized curve defined as in equation 2.

$$S = (x(s), y(s)) \qquad 0 \leq s \leq 1 \tag{2}$$

Furthermore, 'Q' is any point on the parameterized curve 'S' with known coordinates $(\acute{x}, \acute{y})$. The point 'R' with unknown coordinates (x, y) is placed in such a way to make the segment 'RP' be orthogonal to the tangent at point 'Q'. The above requirement commands the equation 3 to be satisfied, where U_{Q-R} is the vector from Q to R and V_{R-P} is the vector from R to P.

$$U_{Q-R}.V_{R-P} = 0 \tag{3}$$

The value of Δt in figure 2 is the Euclidian distance between points Q and R which can be obtained by getting the value of $\sqrt{(x-\acute{x})^2 + (y-\acute{y})^2}$. Furthermore, the point R can be expressed in terms of Δt,the point S_Q and its tangent $\bar{S_Q}$ in the form of equation 4 [7].

$$R = Q + \Delta t * \bar{S_Q} \tag{4}$$

Many logical algorithms have previously been developed to move point Q towards minimizing Δt. One such algorithm [14] attempted to converge the two points based on the equation 4. There, the final resulting point was obtained by inspecting the sign (+/-) of the vector dot product, while traversing in between two end points. In our approach, linear traversing was adopted and starting from one end (randomly selected) of the centerline, the point Q was traversed by a distance $\Delta t/2$ along the centerline. The value of $\Delta t/2$ was selected to assure fast convergence towards $R_(x, y)$ while avoiding overshooting the actual result by having too high a step value.

The convergence was set to be detected by setting a threshold for the Δt value. But, due to high shape variability of chromosomes, this method did not guarantee the global solution, especially on chromosomes which have a bend angle of about 80^0 or above. Therefore, in order to ensure the validity of the projected point result, we proposed using 'Nearest Neighbor'(NN) classification to confirm the accuracy of the iteration result. Nearest neighbor is a very intuitive classification method in which the user presumably has very little or no knowledge regarding the distribution of the test data. In our approach, the NN method was utilized to classify one or more points from the centerline which were the closest to the point to be projected. One of the main drawbacks of this method was the mere possibility of getting more than one point from the centerline as the closest point. Thus, in order to use NN for finding the benchmark point on the centerline, the nearest points (in the case of multiple points) were sorted according to the index and the median of this series of points was obtained.

Next, the difference between the resulting point obtained through initial iteration method and the NN result was computed. If this difference was larger than a pre-determined threshold (empirically set to 4 for this experiment), the geometric iteration method was carried out starting from the

other end point of the chromosome. The final decision was based on both iterations in that case and the projected point was selected based on minimum difference between the NN method and the iteration results. The median point of the NN result was not necessarily the point of interest in our case but served merely as a good first approximation for the result. This algorithm for point projection is illustrated in the flow chart in figure 3,

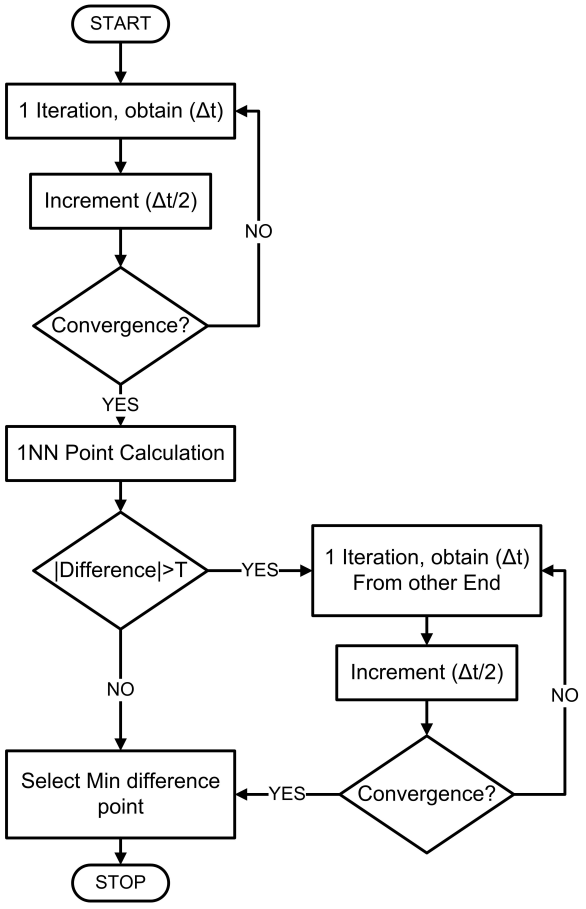


Figure 3. The flow chart of the point projection method used in the research.

3. Results and discussion

A novel approach for accurately detecting FISH probe locations with respect to metaphase chromosome landmarks in fluorescent microscopy images was presented in this paper. Also, it was noted that the accuracy of the extracted centerline as a landmark, heavily determines the outcome of any other measurement result on the chromosome. Therefore, we first tested the accuracy of the centerline extracted through our method against those of the thinning method in [11]. The proposed algorithm was tested on 66 chromosomes extracted from inverted DAPI stained chromosome images captured using an epifluorescence microscope. The chromosomes selected for the DCE based methodology did not overlap or touch each other and they met the minimum length criteria of 35 points (see section 2.2). The chromosomes used in this analysis came of three different individuals from six lymphocyte cells on four microscope slides. The centerline extracted using our algorithm was compared with that obtained through a thinning approach [11]. Two geneticists identified which method was better for identifying the centerline of chromosomes. Preliminary results on the expert assessment are shown in Table 2. These results show that the DCE based method performs either equivalent or better than thinning in nearly all instances. Results using DCE were independent of the source of the chromosomal material, i.e. of the patient, slide or cell that was selected. The accuracy of the DCE based method was particularly high in regions of chromosome bends, which occur more frequently in longer chromosomes. Longer chromosomes are well represented in groups A,B & C in table 2, where the improvements of the DCE based method were apparent. In addition, longer chromosomes are found in pro metaphase chromosomes that are just beginning to condense in mitosis. Another category of relatively longer chromosomes can include chromosome rearrangements such as duplications or translocations.

Table 2. Comparative scoring of DCE vs thinning algorithms - where each value gives the number of chromosomes for which, the centerline was better represented by the corresponding algorithm

Class	DCE	Thinning	Both	Total
A	7	-	5	12
B	5	-	5	10
C	19	-	9	28
D	3	1	4	08
E	6	-	2	08

The results depend on parameters of two stages: namely, the GVF segmentation and the sampling point selection. Firstly, the segmentation outcome was observed to be highly sensitive to the values set for the main internal parameters of the GVF snake such as α (elasticity factor), β (rigidity factor), μ (GVF regularization factor) and κ (external force weight). A sensible set of values for the above factors ($\alpha = 0.05,\ \beta = 0,\ \kappa = 2,\ \mu = 0.2$) provided satisfactory results in our experiments but fine tuning was possible through adjustment. Next, the selection of the

sample point spacing and the starting and ending sample point offset (from the DCE result) were observed as critical parameters in the medial axis extraction and values were set based on empirical observations.

The centerline through our algorithm was also observed to be able to successfully handle bent chromosomes (as seen in Fig 4) and extracted centerline that closely represented the shape information of the chromosome. The sharpness of the centerline results in Fig 4 is mainly due to the pixelation effect of the magnified image.

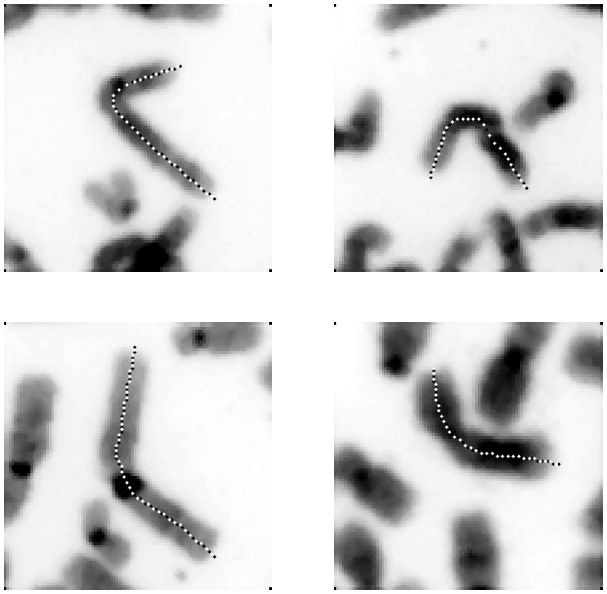
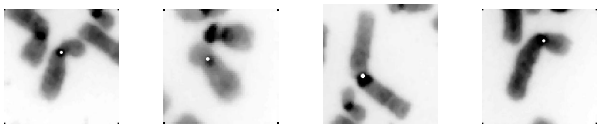


Figure 4. Centerline results of representative bent chromosomes using the proposed approach.

A preliminary experiment was carried out to test the centromere detection process in which, 41 chromosomes were extracted from the same sources used for the centerline comparison. Figure 5 presents typical results produced by the centromere detection process along with respective chromosome groups. Two geneticists identified the accuracy of the detected centromere location and quantified their decision into 3 different categories, namely: 'accurate', 'neighboring' and 'inaccurate'. If the detected centromere location is within 1 chromosomal band distance from the actual centromere location, the 'neighboring' label was assigned. The label 'inaccurate' was accompanied when the error is higher than 1 chromosomal band. Out of the 41 tested cases, only 4 was labeled as 'inaccurate'. 25 cases were labelled as accurate and 12 cases were labeled as neighboring. These results did not depend on the chromosome type (acrocentric, sub-metacentric and metacentric) as well as on the origin of the chromosome.

A preliminary analysis of probe detection by point projection was also carried out. A test probe position was arbitrarily assigned on a chromosome to observe and compute the projected point on the centerline. The initial testing of the point projection on chromosome images yielded satisfactory results (refer figure 6).

The confidence interval circumscribing the predicted centromere will be used during probe localization to weight the contribution of this feature during chromosome abnormality detection. Probe localization on chromosomes with low confidence centromere placement could be biased towards the relative distances to the termini of the chromosome. Alternatively, a model-based approach for centromere analysis can be combined with these confidence intervals. This would involve testing the chromosome-specific probe for detecting a sequence in its normal context, where the expected location of the centromere is already known.



(a) Group C (b) Group C (c) Group A (d) Group B

Figure 5. Some chromosome centromere detection results with their respective chromosome groups.

4. Conclusion and future work

An algorithm that utilizes GVF active contours, DCE based skeleton pruning and morphological thinning is presented in this paper which locates probe signal in relation to chromosome landmarks. Future work includes a suitable method for splitting overlapping chromosomes along with more improvements for the end point correction method to make this algorithm applicable to any metaphase chromosome. Also a refining step needs to be carried out to obtain more accurate centromere locations and a methodology needs to be formulated to reflect the confidence in the refined centromere location.

The proposed algorithm is robust against image boundary noises as well as the high variability of the chromosome shapes. The ability to project FISH probe signals to an accurate centerline approximation, is an important stage in developing a computer based setup to assist clinical diagnosis. Our algorithm can be readily adopted for FISH probe signal localization on chromosome images.

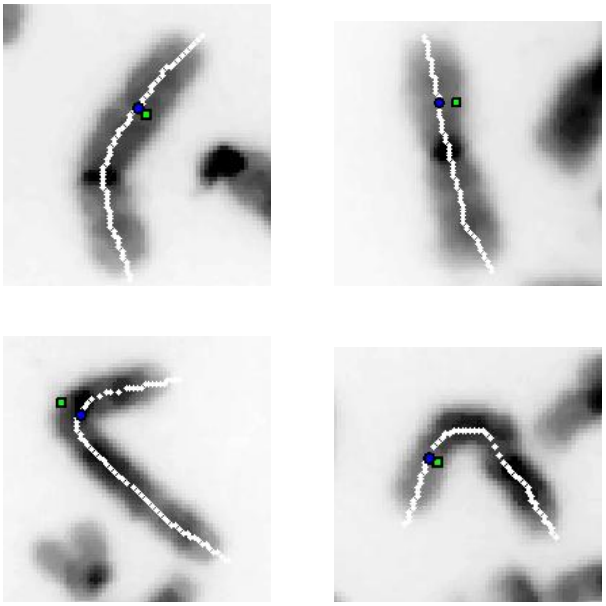


Figure 6. Point projection results - (square - test point : circle - projected point).

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