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Spatial Quantitation of FISH signals in Diploid versus Aneuploid Nuclei

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Abstract

Fluorescence in situ hybridization (FISH) is the most widely used molecular technique to visualize chromosomal abnormalities. Here, we describe a novel 3D modeling approach to allow precise shape estimation and localization of FISH signals in the nucleus of human embryonic stem cells (hES) undergoing progressive but defined aneuploidy. The hES cell line WA09 acquires an extra copy of chromosome 12 in culture with increasing passages. Both diploid and aneuploid nuclei were analyzed to quantitate the differences in the localization of centromeric FISH signals for chromosome 12 as it transitions from euploidy to aneuploidy. We employed superquadric modeling primitives coupled with principal component analysis to determine the 3D position of FISH signals within the nucleus. A novel aspect of our modeling approach is that it allows comparison of FISH signals across multiple cells by normalizing the position of the centromeric signals relative to a reference landmark in oriented nuclei. Using this model we present evidence of changes in the relative positioning of centromeres in trisomy-12 cells when compared to diploid cells from the same population. Our analysis also suggests a significant change in the spatial distribution of at least one of the FISH signals in the aneuploid chromosome complements implicating that an overall change in centromere position may occur in trisomy-12 due to the addition of an extra chromosome. These studies underscore the unique utility of our modeling algorithms in quantifying FISH signals in three dimensions.

Keywords

3D modeling; FISH; aneuploidy; hES cells; superquadric primitives; spatial quantitation of centromere position; centromere position; trisomy-12

Introduction

Fluorescence in situ hybridization (FISH) methodology allows visual detection of numerical and structural aberrations in chromosomes, and has been widely used in clinical cytogenetics for the screening and diagnosis of chromosome abnormalities, such as aneuploidy, polyploidy, deletions, inversions, duplications, and translocations (1). Broadly, FISH approaches can be classified according to the DNA probes used, into repetitive-sequence (centromeric or telomeric), site-specific (loci), or whole chromosome painting (wcp), which has enabled the application of FISH for the delineation of the nuclear positioning of

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genomic regions (2), genes (3), and chromosomes (4), respectively. Studies employing FISH for examining nuclear organization use either 3D-FISH (fixation with buffered formaldehyde), or 2D-FISH (hypotonic treatment with 3:1 methanol/acetic acid) for sample preparation (5), followed by wide field epifluorescence with image deconvolution, or confocal microscopy for data acquisition (6).

Over the past few decades numerous studies employing FISH have unraveled the non-random distribution of chromosomes within the nucleus (reviewed in (7)), and the focus is now on elucidating the role of nuclear architecture in human disease (8). Although the underlying causes of chromosomal instability in cancer are still largely unknown, alterations in chromosomal copy numbers are amongst the most common aberrations frequently encountered in tumors (9). Differences in the degree of chromosomal organization have been noted between normal and tumor cells as indicated by differences in the radial positioning of genes (10). Also it is known that alteration in gene expression mostly ensues in aneuploidy (11), but results on whether any changes occur in chromosome positioning associated with aneuploidy are conflicting. Cremer et al., 2003 indicated alterations in chromosome positioning in tumor cells (12), whereas conflicting findings that chromosomes assume a 3D-position similar to that of their endogenous homologues were reported for artificially introduced chromosomes (13). Others have shown that physical proximity facilitates rearrangements between chromosomes (14,15).

In order to further investigate the role of spatial organization in health and disease, integrative studies combining molecular biology, cytogenetics, bioinformatics and computational modeling are warranted. A comprehensive review of the computational methods and problems for the spatial quantitative analysis of nuclear organization provided by Ronneberger et al. 2008 (16), and a recent review on genome organization by Rajapakse et al. 2011 (17), both highlight the lack of mathematically comprehensive methods to determine the 3D positioning of genomic regions, genes and chromosomes.

Sophisticated algorithms that allow the quantitation of FISH signals are essential to permit the accurate localization of genes and their products in cells and tissues. Accordingly, several novel image analysis tools have been proposed to allow the segmentation, assessment, and visualization of 3D FISH signals (18–21). In this study, we present a 3D modeling approach for estimating the *surface* of nuclei and the enclosed FISH signals. We employed 2D-FISH using centromeric probes followed by confocal microscopy to acquire 3D images of FISH signals, and used superquadric modeling primitives to estimate the surface of both nuclei and the enclosed FISH signals. Despite the flattening of nuclei and FISH signals observed in 2D-FISH, studies have found the radial nuclear distributions of chromosomes to be stable across the 2D-FISH (cells cytopinned onto slides (5), dropping cell onto slides and air drying (22,23)), and 3D-FISH samples (5,22,23).

Previous studies have characterized positioning of genomic components in terms of (1) radial positioning based on a ratio between the center and border of the nucleus (6,12), and (2) relative positioning with respect to other chromosomes (24), or to landmarks such as the nuclear envelope or nucleolus (25). In this study, we present a new computational 3D image analysis framework to determine the specific spatial location of FISH signals, with respect to another single FISH signal designated as a landmark. Due to the inherent absence of unquestionable structural landmarks in nuclei, it is not possible to define the position of a genomic region in absolute terms, and most studies present probability maps of how genomes are spatially organized. We present an approach to specify the 3D position of FISH signals in terms of a coordinate location (i.e. (x, y, z) value) relative to a single predefined peripherally located FISH signal as a landmark within oriented nuclei. This is a novel

feature that directly addresses the issue of using shape-based nuclei registration for analyzing nuclei architecture (26).

The 3D modeling tools developed were validated on simulated data, and used to assess and quantify FISH signals in diploid versus aneuploid nuclei. The centromeric region was chosen to demonstrate the feasibility of the tools developed. Although centromere positioning is not a precise descriptor of chromosome position, it is indicative of a chromosome's global position within the nucleus because centromeres should, by definition, lie within or near the chromosome territory from which they derive. Studies have shown that centromeres exhibit cell type specific interphase patterns (27). Interestingly in sperm cells, it has been shown that centromere topology may be changed as a result of aneuploidy (28).

Spatial positioning of the centromeres of two chromosomes (X and 12) in hES cell line WA09 (29), was studied using dual color 2D-FISH in conjunction with confocal imaging. Our results indicate that in hES cells with trisomy-12, a change in positioning is observed between the centromere groups of chromosome 12 in aneuploid cells when compared to diploid cells in the same population. This study demonstrates the feasibility of using parametric surface estimation for enabling the analysis of the relative distribution of FISH signals within the nuclear volume. Future studies will examine the extension of the tools for applicability to the analysis of other genome regions such as chromosomes and genes.

Materials and Methods

Cell Culture

The hES cell line WA09 (H9) at passage 29 was obtained from WiCell Research Institute (Madison, WI), and the undifferentiated cells were cultured on a mouse embryonic fibroblast feeder layer, strictly following the culture conditions suggested by WiCell, i.e. sub-culturing once a week; over 14 months until passage 87. In order to assess acquired aneuploidy over serial passaging, cultures from every other passage were monitored for undifferentiated cells using a rapid staining assay for alkaline phosphatase activity and/or FACS analysis of dual label SSEA1/SSEA4, and Oct4 as recommended by WiCell. In addition, differentiated cells were manually removed from culture during passaging using the protocol suggested by WiCell. Both morphologically and physiologically, the differentiated cells are distinct from the pluripotent hES cells. In this study, we used only the undifferentiated hES cells, since changes in nuclear architecture have been noted in differentiated cells (27). These changes are indeed related to changes in the distinctive localizations for chromosome regions and gene loci with a role in pluripotency. Wiblin et al., 2005 (27) found that 12p, a region of the human genome that contains clustered pluripotency genes including NANOG, has a more central nuclear localization in embryonic stem cells than in differentiated cells. Also a smaller proportion of centromeres located close to the nuclear periphery in hES cells compared to differentiated cells. Therefore, differentiated cells were removed from the culture. For each passage cell karyotype was analyzed by conventional G-banding, FISH (using centromeric probes for chromosome 12 and 17) and SKY methods. The diploid WA09 cells progressively became aneuploid between passages 35 to 87 by gaining an extra copy of chromosome 12. With increase in passage the percentage of trisomy-12 cells increased and reached approximately 70% by passage 68 and then remained constant until the culture was terminated at passage 87. Forty nuclei were sampled from the passage 34 (hESp34) to provide the baseline control for diploid nuclei, whereas 69 diploid and 66 trisomy-12 nuclei were imaged from the passage 73 (hESp73) cell population.

FISH Slide Preparation and Image Acquisition

Nuclei from passage 34 and 73 were prepared for 2D-FISH using standard protocols as follows. Cells were harvested after reaching 60% confluency. Colcemid (KaryoMAX®, Gibco) at a concentration of 10 µg/mL was added 3h prior to harvesting. In order to verify the hybridization efficiency and to determine the frequency of aneuploid cells (as the hESWA09 cell line transitioned to trisomy-12 with increasing passage numbers), the cultures were treated with colcemid so that in addition to interphase cells, a sufficient number of metaphases would also be available for analysis. Cells collected by trypsinization were treated with 0.38% KCl (hypotonic) for 20 min at 37°C, fixed in methanol: acetic acid (3:1), and dropped onto clean slides. To minimize variations in slide preparation, samples were consistently dropped from a fixed height and at a fixed angle. Dual color FISH was performed on chromosomes obtained from hES (WA09) cells using FITC and Cy3-labeled centromeric probes for chromosome X and 12, respectively (Genetix USA Inc., Boston, MA, USA.). Hybridization and detection were performed according to the manufacturer's protocols. The nucleus was counter-stained with DAPI. Confocal images were acquired on an Olympus FluoView 1000™ system (Olympus Corp. of the Americas, Center Valley, PA, USA) with a 63X Plan-Apochromat 1.4 NA oil immersion objective using a digital zoom of 2 or 3. The zoom function on the Olympus FV1000 (and most other confocal microscopes, as well) controls how wide of an area the laser is scanning while the images are being constructed. The zoom ratio only affects X-Y voxel dimensions with the Z-dimension being controlled independently solely by adjusting the Z-step size. The increased resolution assists in improved delineation of the FISH signals in the XY-plane. Optical sections were acquired at 512×512 pixels per frame using 12-bit pixel depth for each channel at a voxel size of 0.07 × 0.07 × 0.04 µm. The z-stacks were acquired sequentially in a three-channel mode, using the 405, 488, and 543 nm laser wavelengths. Figure 1A shows the maximum intensity projection of confocal optical sections through a trisomy-12 nucleus (blue), depicting centromeric signals for chromosomes X (green) and 12 (red).

3D Segmentation

The serial sectional images were pre-processed by median filtering (radius=2 pixels) to smooth the data. Background shading and spectral bleed-through between the three-color channels was performed, if needed; as previously described (30). Manual gray level thresholding was applied to generate a binary image stack, for each channel (red, green, blue). Figure 1B shows a representative thresholded image for a single optical slice from the blue channel (nucleus). Comparing the segmented objects to the original images via image overlays visually validated the efficiency of resulting segmentations. Recently several thresholding algorithms have been developed for 3D segmentation of cells, nuclei, or genomic regions and loci (18–20, 31–33). In this study, we chose to implement user-defined thresholding, which is generally considered the gold standard for segmentation (26,34,35). Next the centromere signals (X labeled green, and 12 labeled red) within the nucleus were detected using 3D region labeling (26-connected neighborhood), as published earlier (36,37). Boundary points were selected for objects in each of the 3 color channels (i.e. nucleus in blue, X in green and 12 in red) (see Figure 1C), using an edge-detection algorithm (38). The complete set of boundary points for each segmented object consisted of voxels, which lie on the periphery of the segmented region in each interior slice, with the exception of the topmost and bottommost slices, wherein all the points in the segment region were selected as the boundary points. Finally, non-linear least squares minimization (MATLAB, Mathworks, Natick, MA) of the data was performed to estimate the superquadric surface as described below.

Superquadric Modeling Primitives for Estimation of 3D Shape

Superquadrics include the parametric forms of quadric surfaces such as the superellipse or superhyperbola, and are represented by an inside-outside function, or the cost function, which divides the 3D space into three distinct regions: inside, outside, and surface boundary. In this study, since nuclei are generally oval, spherical or discoidal in shape, we chose the superellipsoidal family of quadric surfaces as the base model. The cost function, $F(x, y, z)$, of a superellipsoid surface is defined by Equation (1) below (39):

$$F(x, y, z) = \left(\left(\left(\frac{x}{a_1} \right)^{\frac{2}{\varepsilon_2}} + \left(\frac{y}{a_2} \right)^{\frac{2}{\varepsilon_2}} \right)^{\frac{\varepsilon_2}{\varepsilon_1}} + \left(\frac{z}{a_3} \right)^{\frac{2}{\varepsilon_1}} \right)^{\varepsilon_1} \quad (1)$$

where x, y and z are the position coordinates of points in 3D space; a_1, a_2, a_3 define the superquadric size, i.e. radius along the x, y and z directions, respectively. ε_1 defines the shape along the z -axis, whereas ε_2 defines the shape in the XY plane. A superellipsoid can take various shapes depending upon the values of the shaping parameters ε_1 and ε_2 . For example, if $\varepsilon_1 = \varepsilon_2 = 1$, and $a_1 = a_2 = a_3$, we get the definition of sphere. Equation 1, describes the cost function in the object-centered coordinate system, and can be transformed to the center of the world coordinate system (denoted by the subscript W) following translation and rotation (40). The cost function in the general position is thus defined as follows:

$$F(x_W, y_W, z_W) = F(x_W, y_W, z_W; a_1, a_2, a_3, \varepsilon_1, \varepsilon_2, \phi, \theta, \psi, c_1, c_2, c_3) \quad (2)$$

where $a_1, a_2, a_3, \varepsilon_1$ and ε_2 are described earlier; ϕ, θ, ψ represent orientation in terms of Euler angles, which are defined as the rotation by an angle ϕ about the z -axis, followed by a rotation of angle θ about the x -axis, and finally a rotation of angle ψ about the transformed z -axis; and c_1, c_2, c_3 define the position in space given by the centroid (center of mass). This allows precise estimation of the orientation of the imaged nuclei at any position on the slide.

Additionally, we included estimation of tapering and bending deformations (41), to further optimize the model to fit global distortions of nuclear shape such as curved surfaces and elongated shapes. Following the inclusion of the deformations, the superquadric is defined by the 11 basic parameters (Equation 2) and 4 deformation parameters (40).

$$F(x_W, y_W, z_W; a_1, a_2, a_3, \varepsilon_1, \varepsilon_2, \phi, \theta, \psi, c_1, c_2, c_3, K_x, K_y, k, \alpha) \quad (3)$$

Tapering deformation is controlled by two parameters K_x and K_y , which define elongation along the x - and y -axis, respectively. The amount of deformation along the x , and y -axis is defined as

$$F_x(Z_s) = \frac{K_x}{a_3} * Z_s + 1 \text{ and } F_y(Z_s) = \frac{K_y}{a_3} * Z_s + 1 \quad (4)$$

where Z_s and a_3 are the z coordinate of a point on the superquadric surface of size a_3 along the z -axis.

Bending deformation is controlled by two parameters; α and k . The angle α defines the bending angle with the horizontal plane of the superquadric, whereas, k defines the curvature of bending. The bending transformation (x_b, y_b, z_b) , for a point (x, y, z) on the superquadric surface is described by the following parameters:

$$\gamma = z_b * k, \quad r = \cos \left(\alpha - \tan^{-1} \left(\frac{y_b}{x_b} \right) \right) * \sqrt{x_b^2 + y_b^2}, \quad \text{and} \quad R = k^{-1} - \cos \gamma * (k^{-1} - r) \quad (5)$$

The transformation is given by

$$x = x_b + \cos \alpha * (R - r), \quad y = y_b + \cos \alpha * (R - r) \quad \text{and} \quad z = \sin \gamma * (k^{-1} - r) \quad (6)$$

The hierarchy of transformations is as follows:

$$\text{Translation (Rotation (Tapering (Bending (3D Points on Superquadric Surface))))} \quad (7)$$

Model recovery was implemented by using 3D data points for the nuclei and FISH signals of centromeres obtained from confocal fluorescence microscopy as input (see Figures 1D–1L). Figure 2 shows an example of estimated superquadric surfaces for the nucleus and the enclosed FISH signals.

Determination of Radial and Edge-to-Edge Distances

Two distances, radial and edge-to-edge were computed for each centromere signal. The radial distance is defined as the distance from the centroid of each centromere to the center of the enclosing nucleus, whereas the edge-to-edge distance is defined as the shortest distance between the edges (boundaries) of two centromeres. As shown in Figure 3A, both the radial and the edge-to-edge distances were normalized with respect to the size of the nucleus to allow comparison across various cells from different samples.

Intra-homologous and Inter-heterologous Distances—The normalized edge-to-edge distances between the estimated surfaces of FISH signals were used to estimate the intra- and inter-centromere distances. Intra-homologous distances were defined in terms of distances between the centromeres of homologous chromosomes, (e.g. the distance between two X centromeres' (X-X)). Inter-heterologous distances were defined in terms of distances between heterologous centromeres (e.g. the distance between centromere X and 12 (X-12)).

For a chromosome class, there is one homologous distance during diploidy and more than one homologous distance during aneuploidy. We used the minimum distance between the centromeres of homologous chromosomes to represent the intra-homologous distance (Figure 3B). Similarly, there are several inter-heterologous distances depending on the number of chromosomes in each class. Thus, the average of the minimum of all the inter-heterologous distances between each homologous centromere of one class with each homologous centromere of the other class was used as the representative heterologous distance for each nucleus (Figure 3C).

Determination of 3D Position in Space

Given the normal variation in the size and shape of nuclei and FISH signals within samples, and their dependency on the sample preparation methods, it is essential to formulate appropriate models, controls and normalizations, to enable statistically meaningful comparisons across multiple nuclei from the same sample, and from different samples for any given cell type (16). Two factors are critical for the quantitative analysis of FISH signals in nuclei, (1) orientation of the nucleus, and (2) a structural landmark relative to which the positioning of each FISH signal can be defined.

Establishing a Common Reference Frame—Nuclei are typically found at different orientations based on how the cells fall on the slide during sample preparation. When comparing the specific 3D position of FISH signals, the orientation of the nucleus is important. For example, in the same nucleus a signal can appear at different positions (e.g. at 45°, 90°, ... etc.) depending on the orientation of the nucleus. We performed principal component analysis (PCA) to realign nuclei along similar orientations. The rationale is that cells (or nuclei) that are similar in shape will give rise to similar principal axis orientations. The basic assumption here is that the nuclei can fall on the slide in a varied number of orientations, and the idea is to orient them based on the overall 3D shape of the nuclear envelope. Thus, although the flattening occurs when the nuclei hit the slide, presumably they will all flatten along the surface that touches the slide. Using PCA will align the nuclei so that the major principal vector of each nucleus is aligned to point along the x-axis; the second principal component is aligned along the y-axis, consequently aligning the minor principal component along the z-axis. Thus, it is highly likely that the minor principle component nuclei will lie along the flattened axis, which will be aligned along the z-axis. Following determination of the principal vectors by PCA, all the nuclei were realigned so that the corresponding Eigen vectors were aligned along the same direction. That is, the major principal vector of each nucleus was aligned to point along the x-axis; the second principal component was aligned along the y-axis, consequently aligning the minor principal component along the z-axis. The estimated superquadric surface for each nucleus was thus defined about the orthogonal axes of the new coordinate system (based on PCA), with its centroid located at the origin of the common reference frame.

Establishing a Structural Landmark—The 3D positioning of FISH signals within the nuclear space was determined relative to a structural landmark. We chose one centromere of X as the structural landmark. A nucleus was randomly selected, and the 3D position of one centromere of X within it was predefined as the position (x_g, y_g, z_g Cartesian coordinates or ρ_g, θ_g, ϕ_g in Spherical coordinates) of the structural landmark (global landmark). Then, for each nucleus in the sampled cell population, the centromere of X occupying a 3D position most proximal to the predefined global landmark position was designated as the local structural landmark.

Selection, Realignment and Scaling of Structural Landmarks

Following realignment of each nucleus along the common reference frame using PCA, a single centromere belonging to the same class (i.e. X) was selected as the local structural landmark, and then realigned and scaled to the predefined position of the global landmark. Since each nucleus can have a varying number of homologs for each of the 24 chromosome classes (e.g. two for euploid, vs. one for monoploid, and three for triploid, etc.), each centromere was first classified into a particular group based on its 3D position within the bounding nuclear superquadric surface as described next.

Chromosomes Class based Categorization of Centromeric Signals—

Centromere signals from chromosomes of the same class were initially categorized into groups based on the level of ploidy. For example, each centromere from the pair of diploid X chromosomes may be categorized as belonging to the group, X-1 or X-2. This categorization was performed based on the assumption that, for a given population of cells a centromere of a given chromosome class occupying a similar position in 3D space is assigned to the same group.

Each FISH signal was characterized by its superquadric surface and centroid that defined its volume and specific 3D position, within the bounding nuclear surface. This position was represented in terms of a vector originating from the origin of the common reference frame

(i.e., centroid of the nucleus) and terminating at the centroid of the fitted superquadric surface for the FISH signal. Thus, for each signal, we have a vector that defines its 3D position in a common reference frame. To assign a centromere to a specific group, a scalar value, i.e. the vector dot product, was computed between its position vector and the vector representing the global structural landmark. A larger magnitude of this dot product indicated closer proximity to the global structural landmark. Based on this value each centromere was assigned to the appropriate group (e.g. 1 or 2 for diploid and 1, 2 or 3 for triploid chromosomes). For example, for chromosome X, the centromere most proximal to the global structural landmark was assigned to group 1 (e.g. X-1), while the second closest centromere was assigned to group 2 (e.g. X-2), and so on.

Structural Landmark—Following centromere categorization, one centromere of chromosome X (X-1) was chosen as the local structural landmark in each nucleus, and the position of its centroid (ρ_l, θ_l, ϕ_l) was noted. Next, in each nucleus the local structural landmarks were realigned and scaled to the predefined global landmark 3D position (i.e. $(\rho_l, \theta_l, \phi_l) = (\rho_g, \theta_g, \phi_g)$) as follows. The position vectors for each local structural landmark were rotated such that they all have the same position as the predefined global position, i.e. $\theta_l = \theta_g$ and $\phi_l = \phi_g$. This aligned all the structural landmarks along the same radial axis, albeit at different magnitudes (i.e., similar θ_l and ϕ_l values, but different ρ_l values). In order to establish equivalence of magnitude, the structural landmarks were also scaled to the predefined 3D position. The scaling factor was computed as the ratio of the normalized distance of the structural landmark (ρ_l) for each individual nucleus to that of the predefined distance (ρ_g) given as ρ_l/ρ_g . This scaling factor was then used to appropriately scale the positions of all the other FISH signals such that they were shifted along the radial axis that they lie on, proportionate to the scaling of the structural landmark. This allowed the characterization and comparison of all the centromere FISH signals from different nuclei relative to the same predefined 3D position (i.e. global landmark).

As shown in Figure 4, the realigned and scaled centroid of each FISH signal was used to represent the position of each centromere in the nucleus relative to the structural landmark. Centroid plots were used to visualize the localization of FISH signals across all nuclei from diploid and aneuploid cell populations.

In order to perform a statistical assessment of the 3D position of the FISH signals, the scalar resolute was determined. The scalar resolute of a vector A in the direction of another vector B is given by $|A| \cdot \cos\theta$, where θ is the angle between the vector A and the vector B.

The 3D modeling tools developed were validated on simulated data, and used to assess and quantify FISH signals in diploid versus aneuploid nuclei (see Supplementary Note and Figures S1–S4).

Results

Nuclei were sampled from a mixed population of diploid (~30%) and aneuploid cells (trisomy-12, ~70%) that were taken from cell cultures at passage number 73 (hESp73). In addition, diploid nuclei from cell cultures at passage 34 (hESp34); i.e. prior to initiation of trisomy-12, were sampled to establish the control for the distribution of FISH signals in a diploid cell population. Figure 1A presents the maximum intensity projection of optical sections obtained through the nucleus of a trisomy-12 hES nucleus (two X's and three 12's, with the estimated superquadric surfaces shown in Figure 1E-1L and Figure 2.

3D Position of Centromeric Signals Relative to the Structural Landmark

Here we present data on the spatial location of centromeres X and 12 in hES cells, based on their relative position in the nucleus. For clarity in visualizing the centromere positions across the different centromere groups and cell populations, the average 3D position in terms of the (x, y, z) coordinates was determined for each of the FISH signals, and is plotted relative to the structural landmark X-1 (Reference) in Figure 5. The corresponding histograms of the distributions of the centroids for the centromeres of X and 12 within the diploid and aneuploid cell populations are included as supplementary information (see Figure S5). Figure 5, shows the relative spatial position for the different groups of centromeres; centromere-X, group 2 (X-2), centromere-12, group 1 (12-1), and centromere-12 group 2 (12-2) in hESp34 Diploid (pre-trisomy diploid cell population) and hESp73 Diploid (diploid nuclei from a mixed cell population of euploid and trisomy-12 cells), and hESp73 Aneuploid (trisomy-12 nuclei). For the hESp73 aneuploid population, the position of centromere-12 group 3 (12-3) is also shown.

Centromeric Signals in hESp34 and hESp73 Diploid Nuclei—As seen in Figure 5 (and Supplementary Figure S5), for hESp34 (diploid) and hESp73 (diploid) nuclei, the position of the FISH signals for X-2, 12-1 and 12-2 are segregated. To assess the significance of this observation, we performed a one-way ANOVA. The null hypothesis was that all the 3 spatial positions were equal. For hESp34, the computed values were found to be $F_{\text{calculated}} > F_{\text{critical}}$ ($20.304 > 3.074$) and P-value (2.774×10^{-8}) < 0.05 , and for hESp73 (diploid) cells, $F_{\text{calculated}} > F_{\text{critical}}$ ($35.996 > 3.04$) and P-value (4.086×10^{-14}) < 0.05 . Thus, for both the diploid cell populations we reject the null hypothesis that the 3D position of X-2, 12-1, and 12-2 are equal. Further, we performed a two-way paired t-test with a 95% confidence interval to compare the position of the individual FISH signals for the three centromeres with each other. As seen in Table 1, for each pairwise comparison in both the diploid cell populations we reject the null hypothesis (P-value < 0.05) that the two means are equal.

Centromeric Signals in hESp73 Trisomy-12 Nuclei—For hESp73 aneuploid nuclei, the position of the FISH signals for 12-1 and 12-3 are segregated, whereas the signals of X-2 and 12-2 appear in close proximity of each other (Figure 5 and Supplementary Figure S5). We performed a one-way ANOVA comparing the 3D position of all the four FISH signals, and two way paired t-tests to assess each individual signal with another. The null hypothesis was that the spatial positions were equal. For ANOVA, the computed values were found to be $F_{\text{calculated}} > F_{\text{critical}}$ ($66.296 > 2.639$) and P-value (0.00) < 0.05 , thus we reject the null hypothesis. As shown in Table 1, for all pairwise comparisons, except that for X-2 and 12-2 (P-value = 0.615), we reject the null hypothesis that the means are equal. Collectively, this analysis suggests, that the 3D position of centromere signals in aneuploid nuclei is changed, in that although a separation of positions is seen for 12-1 and 12-3, the centromere signals for X-2 and 12-2 are found to be in close proximity and are not distinct.

Centromeric Signals for X, 12-1 and 12-2 in Diploidy vs. Trisomy-12—As seen in Figure 5, for the hESp73 aneuploid cells, X-2 and 12-1 occupy positions similar to that of the corresponding centromere groups in the nuclei of diploid cell populations (hESp34 and hESp73 diploid). A one-way ANOVA test (H_0 ; equal means), indicated $F_{\text{calculated}} < F_{\text{critical}}$ ($0.257 < 3.049$) and P-value = 0.774 for X-2, and $F_{\text{critical}} > F_{\text{calculated}}$ ($3.0491 > 1.39$) and P-value = 0.252 for 12-1, thereby failing to reject the null hypothesis. Additionally, we performed a t-test for comparing two means assuming equal variances and 95% confidence interval, with $\alpha = 0.05$, to compare the 3D position of X-2 and 12-1 in the diploid versus aneuploid samples (Table 2). Overall, P-values were non-significant (> 0.05), suggesting that the null hypothesis could not be rejected. These results suggest that position of euploid

centromere group 2 (i.e. X-2) in the aneuploid nuclei (i.e. trisomy-12) is similar to that found in the disomic nuclei, and the position of one of the centromere groups of the trisomic chromosome 12, i.e. 12-1 is also similar across the diploid and aneuploid samples. Contrary results were observed for 12-2, where we failed to accept the null hypothesis in a one-way ANOVA test that the group means are equal across the three (hESp34, hESp73 diploid, and hESp73 aneuploid) populations ($F_{critical} (3.049) < F_{calculated} (14.995)$, $P\text{-value} = 9.93 \times 10^{-7} < 0.05$). Also, a two means t-test with equal variances for comparing each of the three groups pairwise, indicated that 12-2 occupies a similar position in the two diploid cell populations, but in the trisomy-12 samples, there is a change in its position which is found to be significantly different from that in the diploid nuclei (Table 2). Collectively our results suggest that when comparing the three groups of centromeres of chromosome 12 in the trisomy-12 nuclei, with the corresponding two groups in diploid nuclei, one of the groups of centromere-12, retains the same position in both the aneuploid (trisomy-12) and the diploid cell populations, whereas a change is observed in the position of the second group in the trisomy-12 samples, and the third group (trisomy-12 only) is located at a position distinct from the other two groups found in both the diploid and trisomy-12 nuclei.

Radial Localization of Centromeric Signals

A plot of the radial distribution for all FISH signals within both the diploid and trisomy-12 cell populations is shown in Figure 6A, whereas the average radial position is shown in Figure 6B. As seen in Figure 6B, both the centromeres of X, X-1 and X-2 are found to be located at an average normalized radial distance of 0.61 ± 0.2 (nuclear periphery is at a radial distance of 1.0 and the center of the nucleus is at 0.0). Similarly, the three centromeres of 12, 12-1, 12-2, and 12-3 were found to be located at a normalized radial distance of 0.61 ± 0.2 . Statistical analysis using hypothesis testing (ANOVA and t-test) showed no significant differences between the radial distances for all but one pairwise comparison. The only significant difference ($P\text{-value} = 0.016 < 0.05$) was between the radial distance of 12-2 in the diploid hESp34 population and that in the trisomy-12, hESp73 population. This data suggests that overall change in centromere position may occur in aneuploid cells due to the addition of an extra chromosome.

Distances Between Homologous and Heterologous Centromeres

The results of the intra-homologous (X-X and 12-12) and inter-heterologous distance (X-12) between the FISH signals are presented in Figure 7A. For the diploid cell populations of hESp34 and hESp73, the intra-homologous distance for X-X was not significantly different from that of 12-12 (failure to reject the equal means null hypothesis in a two-way paired t-test; hESp34 (diploid): $P\text{-value} = 0.877$, and hESp73 (diploid): $P\text{-value} = 0.091$), and both the intra-homologous distances were found to be greater than the inter-heterologous distance (X-X vs. X-12: hESp34 (diploid); $P\text{-value} = 0.006$, hESp73 (diploid); $P\text{-value} = 4.4 \times 10^{-5}$, and 12-12 vs. X-12: hESp34 (diploid); $P\text{-value} = 0.008$, hESp73 (diploid); $P\text{-value} = 3.5 \times 10^{-10}$). The finding that the intra-homologous distance is greater than the inter-heterologous distance is comparable with previously published results (42). An interesting new finding of this work (Figure 7B) is that in the trisomy-12 nuclei, although the intra-homologous distance for the euploid centromere, i.e. X-X is greater than the inter-heterologous distance X-12 ($p\text{-value} = 0.014$), the intra-homologous distance for the centromeres of the trisomic chromosome, i.e. 12-12 was not found to be significantly different from that of the inter-heterologous distance X-12 ($p\text{-value} = 0.261$). Moreover, an almost 50% reduction in the distance from that seen in the diploid nuclei, was observed between the centromeres for the trisomic chromosome 12 (12-12 hESp34 diploid vs. 12-12 hESp73 Aneuploid; $P\text{-value} = 9.7 \times 10^{-6}$, and 12-12 hESp73 diploid vs. 12-12 hESp73 Aneuploid; $P\text{-value} = 4.22 \times 10^{-8}$). A comparison of the intra- and inter-chromosomal distances between the diploid and aneuploid nuclei is shown in Table 3 validates the significance of this observation. There is no

significant difference in the intra-homolog distance of the centromeres of the euploid chromosome X between the normal and aneuploid samples, whereas there is significant difference between the intra-homolog distances for the centromeres of chromosome 12 in the normal cells versus that in trisomy-12 cells. Collectively these results suggest that aneuploidy results in a reduction in the intra-centromere distance, which may be attributed to the presence of the additional chromosome.

Discussion

We have described a new computational method for the quantitation of the 3D position of FISH signals, and have applied it to compare centromere positioning in diploid versus trisomy-12 nuclei from a hES cell culture model with progressive aneuploidy. Previous methods for quantitation of FISH signals in the interphase nucleus have largely determined organization of nuclear components in terms of radial analysis (43), relative positioning (6), or spatial statistics (44).

The key distinguishing feature of our approach is that the computational framework presented can be used to compare the spatial distribution across multiple nuclei from a given population and/or between populations. Comparing spatial distributions across multiple nuclei is difficult, since nuclei not only vary in shape and size, but also lack landmarks that can facilitate normalization across multiple nuclei (26). Thus, in this study we have proposed the use of the nuclear surface in an innovative way to facilitate the establishment of multiple nuclei into a common coordinate system. A similar idea has been recently proposed by Russell, et al., 2011, wherein they demonstrate the feasibility of using the nuclear envelope as a landmark in order to put nuclei into a common coordinate system, by combining image registration and statistical shape analysis methodology (26). Their approach consists of using 2D intensity projections, followed by identification of mathematical pseudo-landmarks for each nuclear envelope, and subsequent landmark based image registration to create an average nuclear envelope and ultimately the registration of nuclear elements from each image into the average envelope. Despite, the similar premise of establishing a common framework for comparing multiple nuclei based on the nuclear envelope, our approach differs as follows. We determine a superquadric surface to define the nuclear envelope in 3D, and establish a common reference framework using PCA wherein subsequent transformation of the estimated surface allows multiple nuclei to be aligned in similar orientations. This allows us to establish a predefined centromere as the global structural landmark within the common framework, and enables the assessment of the specific (x, y, z) position of FISH signals relative to the landmark. All the conclusions in this paper are drawn based on the methodology described. It is important to note that our approach does highlight key similarities across multiple nuclei that are from different cell populations, which provides proof-of-concept.

3D information on FISH signals across multiple nuclei is scarce in the published literature. Berger, et al., 2008 analyzed the spatial location of a given locus in a coordinate frame intrinsic to the budding yeast, *S. cerevisiae* nucleus (25). The coordinate frame was defined in reference to the yeast nuclear geometry with an oriented central axis' passing through: the spindle pole body on the nuclear envelope, the nuclear center and nucleolar centroid. They determined the 3D position of the locus in terms of its distance from the nuclear center and angle from the oriented central axis. The only other attempt at determining the specific position of FISH signals is the work of Nagele et al., 1999 (45), which looked at positioning in 2D space but did not account for nuclear shape. The significance of determining the specific positioning is the ability to uniquely and independently describe the relative position of each FISH signal within the nucleus. This information differs from characterization in terms of the radial distribution by providing position within an octant of

3D space (analogous to the 4 quadrants in 2D space) for each FISH signal. That is, two signals lying at the same radial distance, but within different octants in 3D space are indistinguishable with radial characterization (which only suggests preferred central vs. peripheral localization), but precisely identified by our approach, allowing delineation of neighboring FISH signals and providing patterns of signal arrangement. Such information would be valuable in performing spatial assessment of gene regulation cohorts.

In our study, samples were processed using 2D-FISH and as expected we observed flattening of the nuclei and signals. However, the use of confocal microscopy enabled the acquisition of 3D data on the flattened nuclei and enclosed centromeric signals, while allowing us to differentiate between the FITC (green) and Cy3 (red) labeled centromeres without residual spectral overlap. Additionally, most microscopy techniques suffer from inherent limitations such as Z-anisotropy wherein the axial resolution is not equivalent to the lateral resolution, chromatic shifts, and intensity attenuation with depth. Techniques such as chromatic shift correction (25), depth based intensity correction (46), and up-sampling in the z-direction (47) are typically used for correction. We have performed minimal data pre-processing limited to median filtering (radius = 2), followed by manual thresholding to generate the 3D point clouds required for surface estimation via superquadric modeling. Overall, all the samples received similar treatment in terms of preparation, acquisition and analysis allowing us to effectively compare the diploid vs. aneuploid nuclei. It should be noted that in this study, the z dimension is reduced due to the 2D-FISH sample preparation procedure utilized. The flattening of nuclei (due to being dropped on the slide) can introduce bias in the determination of distances between centromeres, as the FISH signals appear to be extended along the z-direction. Despite this limitation our analysis tool has identified significant differences in the position of the FISH signals in the control versus aneuploid cells (both populations cultured and prepared for imaging using similar protocols), indicating the robustness of our 3D modeling approach.

We used the tools developed in this study to test the hypothesis that aneuploid states result in altered centromere localization in the nucleus. The human embryonic cell line; hES WA09 (H9), which spontaneously acquires trisomy-12 over serial passaging, was labeled for centromeric FISH signals on chromosome X and 12, and diploid and aneuploid cells from the same cell population were analyzed for determination of spatial organization. We used X as the internal control relative to which the position of 12 could be mapped. The use of chromosome-X as a control reference chromosome has been advocated previously (16), thus using a centromere of X in this study was an appropriate choice. Since, in this study one centromere of chromosome X is used as the landmark, the analyses is based on the assumption that chromosome X is not affected with trisomy-12. This assumption is based on global gene expression analysis of the early diploid and trisomy-12 hES cells. Genome-wide expression analysis of the hES cells at early (Passage 30 diploid) and later passages (Passages 45, 50, 60 and 68 with progressive trisomy-12) was performed using Illumina's HumanWG-6 v3 BeadChips, which contains greater than 48K transcript probes (Pati et al., manuscript in preparation). Analysis of the chromosomal distribution of significantly expressed genes indicated a progressive increase in the copy number of the chromosome 12 genes. A significant decrease in the expression of genes in chromosome 15 and increase in chromosome 17 with progressive trisomy-12 were also noted, but no change in the expression of chromosome X genes was observed.

The FISH signals were characterized in terms of their 3D position in space, radial distance, and intra-homologous and inter-heterologous distances relative to a structural landmark (X-1; group 1 centromere of X). Our results show that in diploid cell populations, centromeres for both chromosomes; X and 12, occupy discrete positions in 3D space. This is

in accordance with previous research that centromeres exhibit cell type specific interphase patterns (27).

An important finding of our study is the confirmation that the nuclear organization of centromeres for the aneuploid chromosome changes in diploid versus trisomic nuclei. Our results demonstrate that centromere X-2 is found in a similar (i.e. non-random) position in both diploid and trisomy-12 nuclei. This is an interesting observation, that warrants future studies to determine whether this finding is true for the other euploid chromosomes (1–11, 13–22). In aneuploidy, although one centromere group of the trisomic chromosome 12 occupies a unique position that is similar to diploid nuclei, there is a change in the position of the other centromere signals. Intriguingly, 12-1 conserves its location (i.e. its positioning is similar to that observed in diploidy-12), but that of 12-2 is changed, and the additional centromere (12-3) establishes a discrete position within the nucleus.

Also we observed that the centromere of one of the trisomic chromosomes (12-2) is found to be in close proximity of one centromere group of chromosome X (X-2) in the aneuploid cell population, an effect that was not observed in both the diploid cell populations. A plausible explanation for this is the change in the position of the trisomic centromere group (12-2) in the aneuploid nuclei (Figure 6B). We note that while the position of a centromere is not an accurate representation of the position of the chromosome territory it occupies, it should closely approximate the global position of its chromosome (45,48), i.e. it is highly unlikely that a centrally located chromosome territory would have a peripherally located centromere.

Next, we found that the intra-homologous distance for both centromeres (X-X and 12-12) is greater than the inter-heterologous distance for centromere X and 12 (X-12), which correlates with that previously observed for chromosomes (42). Probable explanations for this relationship suggest a larger distance between homologs is needed to avoid homologous recombination, and to potentially avoid damaging both copies of a gene by environmental or intrinsic stresses.

An interesting finding is that in the aneuploid nuclei, although the intra-homologous distance for the diploid centromere, i.e. X-X is greater than the inter-heterologous distance X-12, the intra-homologous distance for the trisomic centromere, i.e. 12-12 while not different from that of the inter-heterologous distance X-12, is reduced (by ~50%) compared to the 12-12 distance seen in diploid nuclei. This data suggests that the inter- and intra-centromere relationship is different for the aneuploid chromosome 12, when compared to euploid chromosome X.

In summary, we present unequivocal evidence of changes in the positioning of centromeres of chromosome 12 in hES cells acquiring trisomy-12. In this study, we only analyzed two centromeres (X and 12), and observed changes in centromere positioning for the chromosome 12, which displayed number variations. It would be interesting to evaluate, additional euploid chromosomes in the hES WA09 cell line to determine how the positioning of the other euploid centromeres is affected by trisomy-12. In trisomy-12, within the three groups of centromeres, one group was found to conserve a position similar to that seen for the counterpart group in diploid cells, whereas the second group showed repositioning. It should be noted that in this study, we are not specifically identifying individual chromosomes, to differentiate the maternally derived chromosome homolog from the paternally derived chromosome, or active from inactive. Rather the centromeres are segregated into groups based on the distance from the landmark (i.e., the most peripheral chromosome X centromere). Thus, it is interesting that we observe similarity in the positioning of certain centromere groups between the diploid and aneuploid cell populations. In light of the current consensus that chromosomes and centromeres occupy non-random

positions within the nucleus, these observations are compelling in that while most centromere groups showed position similarity between diploidy and trisomy, one of the aneuploid centromere groups showed a discrepancy in position when compared to the corresponding group in the diploid cells. More experiments are required to test this observation with additional diploid chromosomes, and to also test the potential of using allele genotyping to identify individual homologs of the trisomic chromosomes. Overall this study highlights the proposed method's potential for analyzing spatial organization of FISH signals.

Our data suggests, that centromere localization may be useful in distinguishing normal and tumor cells. Although in this study we applied the 3D modeling to centromeric FISH signals, the tools can be potentially applied to analyze FISH signals from whole chromosome painting allowing precise delineation of chromosome territories (Supplementary Figure S6).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative images for estimation of superquadric surfaces for nuclei and enclosed FISH Signals

(A) Maximum Intensity Projection of confocal optical sections through a trisomy-12 nucleus (blue), depicting centromeric signals for chromosomes X (green) and 12 (red), (B) Result of segmentation for a single optical section (blue channel), (C) Results of edge detection for a single optical section (merged result of red, green and blue channels) (D) Boundary points from the nucleus used for surface estimation, (E) Overlay of boundary points (blue) and line rendering of estimated superquadric surface for the nucleus, (F) Overlay of boundary points (blue) and point rendering (black) of estimated superquadric surface for the nucleus, (G) Boundary points from a centromeric signal for chromosome X used for surface estimation, (H) Overlay of boundary points (green) and line rendering of estimated superquadric surface for a centromeric signal for chromosome X, (I) Overlay of boundary points (green) and point rendering (black) of estimated superquadric surface for a centromeric signal for chromosome X, (J) Boundary points from a centromeric signal for chromosome 12 used for surface estimation, (K) Overlay of boundary points (red) and line rendering of estimated superquadric surface for a centromeric signal for chromosome 12, (L) Overlay of boundary points (red) and point rendering (black) of estimated superquadric surface for a centromeric signal for chromosome 12.

point rendering (black) of estimated superquadric surface for a centromeric signal for chromosome X, (J) Boundary points from a centromeric signal for chromosome 12 used for surface estimation, (K) Overlay of boundary points (red) and line rendering of estimated superquadric surface for a centromeric signal for chromosome 12, (I) Overlay of boundary points (red) and point rendering (black) of estimated superquadric surface for a centromeric signal for chromosome 12.

Figure 2. Three-dimensional surfaces generated via superquadric modeling

Plot of 3D surfaces for a nucleus (blue) and the enclosed FISH signals (two green and three red) generated using the superquadric modeling approach.

Figure 3. Radial, Edge-to-Edge, Intra-homologous and Inter-heterologous Distances

(A) The radial distance of FISH signals was calculated using two different distances. The first distance value is the distance (G_c) between the centroid values of the nucleus and FISH signals, is obtained from the parameters of the respective fitted superquadrics. The second distance (G_f) is the line that connects the centroid of a FISH signal and the centroid of nucleus and intersects the superquadric surface. Estimated surface parameters are used to determine the points of intersection of the 3D lines and the superquadric surfaces. Similarly, the normalized edge-to-edge distance between two signals is computed as the ratio GR_s/GR_f . (B) The minimum distance between homologous signals for a given nucleus was used to represent the intra-homologous distance. For example, the intra-homologous distance for chromosome-12 during triploidy was computed as $d_{hom} = \min(d(12_1, 12_2), d(12_1, 12_3), d(12_2, 12_3))$. (C) There are several inter-heterologous distances depending on the number of chromosomes in each class. For example, in diploid nuclei with two; X- and 12-chromosomes, there are four inter-heterologous distances between the centromeric signals, i.e. X-1:12-1, X-1:12-2, X-2:12-1, and X-2:12-2. The inter-heterologous distance between X and 12 in a diploid nucleus was computed as

$$d_{net} = \frac{[\min(d(X_1, 12_1), d(X_1, 12_2)) + \min(d(X_2, 12_1), d(X_2, 12_2))]}{2}.$$

Figure 4. Realignment along a Common Reference Frame and Structural Landmark

Schematic illustrating realignment of (1) nuclei along a common reference frame and (2) centromeric signals relative to a structural landmark. (A) Plot representing the normalized spatial position of FISH signals in a single nucleus. Four signals representing two classes of chromosomes (X; green icons and 12; red icons) are shown by the patterned oval symbols. The solid circle icon (dashed outline) in pale green represents the global structural landmark. (B) In the next step, a local landmark is identified based on its proximity to the global landmark. As seen in (B), the signal represented by the green oval (checkered pattern) is closest to the global landmark, and is thereby chosen as the local landmark. It is spatially rotated and realigned along the same radial axis as the global landmark. (C) The local landmark is then scaled in magnitude to precisely coincide with the global landmark. Consequently, all other signals are proportionately displaced by the same amount along their radial axis. (D–F) This procedure (A–C) is repeated for all nuclei in a given sample. (G) The 3D spatial position of all FISH signals is then visualized relative to the same structural landmark.

Figure 5. Spatial position of centromeric FISH signals for chromosome X and 12 homologs in diploid and aneuploid nuclei

Positioning in 3D space of the centromere groups for chromosomes X and 12, relative to the reference group X-1 (cross symbol indicates position of X-1). These results show that in diploid cell populations, all the centromere groups for both chromosomes, X and 12 occupy discrete positions in 3D space, with each group occupying its own unique location within the nucleus. Centromere group 2 (X-2) is found in a similar position in both diploid and aneuploid (trisomy-12) nuclei. Additionally, the results indicate that centromere localization is changed during aneuploidy. In trisomy-12 nuclei, out of the three centromere groups only one group (i.e. 12-1) conserves a 3D position similar to that found in diploid 12 nuclei, whereas the position of the second group (12-2) is changed, and the third group (12-3) is seen to occupy its own separate position in the nuclear space.

Figure 6. Radial Analyses of centromeric FISH signals in diploid and aneuploid cell populations

(A) Histogram showing the radial distribution of the centromere groups in chromosomes X and 12. The % frequency of occurrence is plotted against the normalized radial distance from the centroid, binned at intervals of 0.1, with 0 being the center and 1 representing the periphery. (B) Average radial distance from the center of the nucleus. The graph shows that both the groups X-1 and X-2 are found to be located at a normalized radial distance of 0.61 ± 0.2 . Similarly, the three groups of 12-1, 12-2, and 12-3 are found to be located at a normalized radial distance of 0.61 ± 0.2 . Error bars represent standard deviation.

Figure 7. Intra-homologous and inter-heterologous distances between centromeric FISH signals for X and 12

The intra-homologous (X-X, and 12-12) and inter-heterologous distance (X-12) between the FISH signals, were computed as the normalized edge-to-edge separation distance between the signals. (A) Plot of the distances across the diploid and aneuploid cell populations. As seen in diploid cells, the intra-homologous distances are larger than the inter-heterologous distances. In the aneuploid cell population, although the intra-homologous distance X-X is larger than X-12, there is a reduction in the intra-homologous distance 12-12 relative to X-12. (B) It can be seen that the distance X-X and X-12 are similar across all the cell populations, while the distance 12-12 is reduced in the trisomy-12 cell population. Error bars represent standard deviation.

Table 1
Paired t-test: 3D position of centromere groups in diploid and aneuploid cell populations.

Ch	hEsp34 Diploid			hEsp73 Diploid			hEsp73 Aneuploid			
	X-2	12-1	12-2	X-2	12-1	12-2	X-2	12-1	12-2	12-3
X-2	1.00			1.00			1.00			
12-1	0.037	1.00		8.4×10^{-6}	1.00		1.6×10^{-7}	1.00		
12-2	0.001	3.4×10^{-7}	1.00	0.002	0.000	1.00	0.615 [§]	6.7×10^{-16}	1.00	
12-3	NA	NA	NA	NA	NA	NA	5.3×10^{-10}	0.000	4.4×10^{-16}	1.00

[§]Non-significant differences (P>0.05).

Using a paired two-way t-test for each pairwise comparison for the diploid cell populations we reject the null hypothesis (P-value $\ll$ 0.05) that the two means are equal. These data corroborate previous observations that in a diploid cell population of the same cell type, centromeres occupy non-random positions in the nucleus. For the aneuploid cell population, with the exception of the comparison of X-2 and 12-2 (P-value = 0.615); we reject the null hypothesis that the means are equal. This suggests, that the position of a centromere group in aneuploid nuclei is changed, in that although a separation in position is seen for 12-1 and 12-3, X-2 and 12-2 are found to be in close proximity.

Table 2

Position of centromere groups in diploid versus aneuploid samples.

X-2				
Sample	Size	hESp34 Diploid	hESp73 Diploid	hESp73 Aneuploid
hESp34 Diploid	40	1.000		
hESp73 Diploid	69	0.638 [§]	1.000	
hESp73 Aneuploid	66	0.923 [§]	0.5 [§]	1.00
12-1				
hESp34 Diploid	40	1.000		
hESp73 Diploid	69	0.404 [§]	1.000	
hESp73 Aneuploid	66	0.097 [§]	0.345 [§]	1.00
12-2				
hESp34 Diploid	40	1.000		
hESp73 Diploid	69	0.221 [§]	1.000	
hESp73 Aneuploid	66	2.4×10^{-6}	1.7×10^{-5}	1.00

[§]Non-significant differences (P>0.05).

The student's t-test (Two tail homoscedastic at 95% confidence interval) was performed for comparing position of X-2, 12-1 and 12-2 in diploid versus aneuploid samples. P-values > 0.05 were computed for all the group-wise comparisons. These results show that X-2 and 12-1 are found in similar locations across diploid and aneuploid (trisomy-12) nuclei, respectively. However, while 12-2 is found in similar locations in the two diploid cell populations, its position is changed in aneuploidy (trisomy-12) nuclei. This data not only corroborates with previously published evidence that for a given cell type, centromeres exhibit a non-random distribution within the nucleus, but also brings forward a new observation that certain groups of centromeres in aneuploid nuclei for chromosomes showing copy number variations may exhibit repositioning.

Table 3

Intra-homologous and inter-heterologous distances in normal versus aneuploid samples.

Distance	P-value
hESp34: X-X vs. hESp73 diploid: X-X	0.326 [§]
hESp34: X-X vs. hESp73 aneuploid: X-X	0.110 [§]
hESp73 diploid: X-X vs. hESp73 aneuploid: X-X	0.412 [§]
hESp34: 12-12 vs. hESp73 diploid: 12-12	0.768 [§]
hESp34: 12-12 vs. hESp73 aneuploid: 12-12	9.7×10^{-6}
hESp73 diploid: 12-12 vs. hESp73 aneuploid: 12-12	4.2×10^{-8}
hESp34: X-12 vs. hESp73 diploid: X-12	0.061 [§]
hESp34: X-12 vs. hESp73 aneuploid: X-12	0.181 [§]
hESp73 diploid: 12-12 vs. hESp73 aneuploid: 12-12	0.475 [§]

[§]Non-significant differences (P>0.05).

P-value for statistical difference between intra-homologous and inter-heterologous distances between normal and aneuploid samples. The statistical significance was assessed using a Student's T-Test (Comparison of two means with equal variances and $\alpha = 0.05$).