Interactive Algorithms for Rapid Enumeration of Hybridization Signals in Individual, Whole Cell Nuclei Inside Intact Tissue Specimens

Stephen Lockett[1], Curtis T. Thompson[2], James Mullikin[1], Damir Sudar[1], Ramin Khavari[3], William Hyun[3], Daniel Pinkel[1], Joe Gray[1]

[1]Resource for Molecular Cytogenetics, MS 74-157, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. [2]Division of Dermatopathology, Department of Pathology, [3]Division of Molecular Cytometry, Department of Laboratory Medicine, The University of California, San Francisco, California 94143

ABSTRACT

Fluorescence in situ hybridization (FISH) is useful for analyzing specific nucleic acid sequences in individual cells. Its application to tissue sections has been limited however because of the difficulties of performing the hybridization and analysis in sections that are thick enough to contain intact nuclei. Recent improvements in FISH permit hybridization with chromosome-specific, centromeric probes throughout 20 μm formalin fixed, paraffin-embedded sections, which do contain many intact nuclei. This paper describes software to facilitate analysis of these 3D hybridizations.

We have developed two algorithms for analyzing 3D, confocal images of thick sections. One displays 2D, maximum-intensity, projection images through the original 3D image at different angles. When projections are viewed sequentially, the 3D image appears semi-transparent and rotates. The second algorithm allows interactive enumeration of FISH signals. Each signal is marked by the analyst. Then, for each pair of marked signals, a 2D slice image along the line connecting both marked signals and parallel to the z (depth) axis is displayed. From this slice, the analyst decides if the signals are in the same or different nuclei, or if the signals should be rejected because they are in a nucleus truncated by the upper or lower surface of the section. After consideration of all pairs of signals, the algorithm produces a map of the tissue section showing the numbers of signals in each of the intact nucleus.

The algorithms enable analysis of small, premalignant and early malignant lesions and infiltrative lesions that cannot be analyzed by other molecular techniques and permit the direct correlation of FISH information with histology/cytology.

Key words: fluorescence in situ hybridization, confocal microscopy, 3D image analysis, chromosome

1. INTRODUCTION

Genetic aberrations play an important role in the initiation and progression of cancer. Some aberrations manifest themselves as changes in chromosome copy number (aneusomy). Such changes have frequently been detected in cancers and are potential prognostic markers. However, there is little known about the level of these aberrations in tumors or about mechanisms by which they occur. Fluorescence in situ hybridization (FISH) with chromosome specific probes provides information about chromosome and DNA copy number. It is necessary to analyze FISH signals within intact nuclei in order to precisely enumerate the aberration in each nucleus. Analysis of intact tumor sections allows correlation of cytogenetic and histologic parameters which may be critical in the study of premalignant, early and infiltrative tumors.

The combination of FISH to label the centromeres of specific chromosomes, followed by microscopy and digital image analysis is a promising approach to analyze genomic aberrations on a nucleus by nucleus basis. It has been used to enumerate FISH signals in the individual nuclei of thin (4 μm) tissue sections[1,2]. However, accurate results cannot be expected because nearly all the nuclei are truncated in these sections. A solution is to use thicker (e.g. 20 μm) sections, which contain many intact nuclei, followed by 3D image acquisition using confocal microscopy and 3D image analysis to identify the intact nuclei and quantify the aberrations within them. FISH techniques for labeling 20 μm sections followed by confocal imaging have already been reported[3]. However, 3D image display and analysis techniques for efficient enumeration of FISH signals in individual nuclei in intact tissue have not been developed.

The purpose of this study was to develop software algorithms for conveniently displaying 3D images of FISH signals in thick tissue sections and for efficient, interactive enumeration of the signals on a nucleus by nucleus basis within the intact section.

2. METHODS

2.1 3D image display

We developed a simple and fast algorithm for displaying 3D confocal images of thick (20 μm) sections double labeled with a chromosome specific FISH probe and a fluorescent nuclear stain (e.g. propidium iodide, PI). For each label, the algorithm prepares a "projection" image through its 3D image. The projection image has the same (x, y) pixel coordinates as the 3D image, and the intensity of its pixels is the maximum intensity along the line through the 3D image at the same (x, y) coordinate and parallel to the z-axis (fig. 1). The z coordinate of the pixel with the maximum intensity is also recorded in a look up table (LUT) for later use when interactively marking FISH signals (§ 2.2 below). Projection images from the probe and nuclear stain are combined into one color image, where red represents the nuclear stain and green the FISH label. The result is that all high intensity signals, i.e. those from FISH signals and nuclear staining, are shown in the projection image regardless of their depths in the 3D image. The only exception is when two signals are directly on top of each other in the 3D image (i.e. they have the same (x, y) coordinates). In this situation, only the brighter signal is projected into the projection image and the other signal is not visible. Such overlapping signals can be seen by examining projections prepared after rotating the original 3D image (fig. 1). A series of projection images at 10° rotation intervals is prepared. When these image are viewed sequentially, the 3D image appears semi-transparent and rotating.

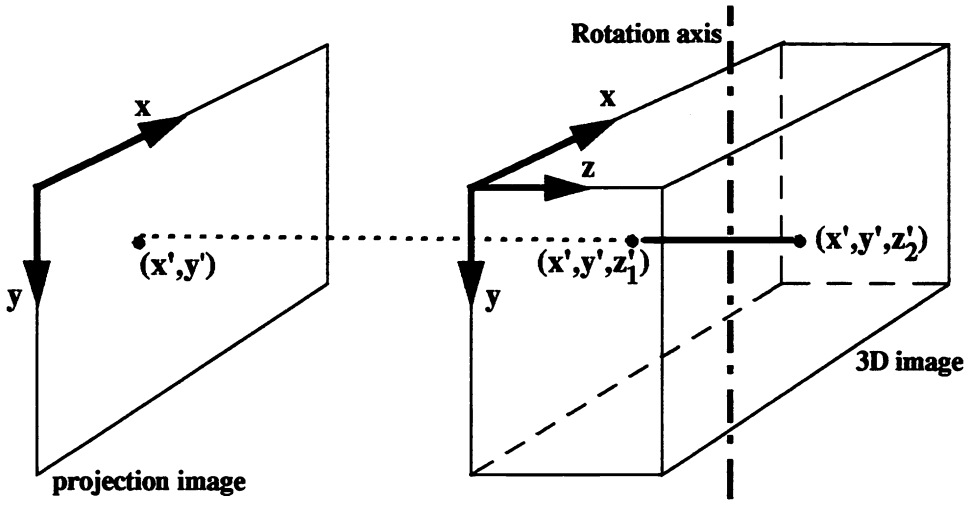

Fig. 1. Preparation of the projection image. The intensity at (x',y') in the projection image is the maximum intensity along the line $(x',y',z'_1;x',y',z'_2)$ in the 3D image.

2.2 Enumeration of FISH signals in individual nuclei

Interactive enumeration of FISH signals in the individual, intact nuclei of tissue specimens is accomplished in two stages. In the first stage, an algorithm enables the analyst to label all the FISH signals in a projection image by marking on a pixel within the signal with the computer's mouse. The color of each marked pixel changes to purple. After marking all signals visible in one projection image, a second projection image, prepared from a rotated version of the 3D image, is displayed. Marked pixels in the first projection image are always projected into the second projection image and are shown in purple. If spots overlapped in the original projection image, they are separated in the second projection. One of the spots would have been marked previously in the first projection and will therefore be shown marked in purple in the second projection. The analyst now has the chance to mark the previously concealed spot. For confirmation, the analyst can inspect any of the x/y 2D slices constituting the 3D image with the marked pixels shown in purple. It is also possible to mark pixels directly on a 2D slice. However, since FISH signals generally extend over several slices, it requires inspection of multiple 2D slices to identify the brightest pixel within each signal. Furthermore, a signal

shown as marked in one 2D slice will not be shown as marked in other slices, even if its corresponding signal is still visible. The (x, y, z) coordinates of all the marked pixels in the 3D image are recorded.

In the second stage of enumeration, each pair of marked FISH signals closer than a user-defined distance (typically 1.5 nuclear diameters) are displayed, and the analyst is asked if the signals are in the same nucleus, different nuclei, or if one or both signals should be rejected. To enable the analyst to make the decision, three images containing the pair of signals under consideration are displayed. The first is the projection image prepared from the unrotated 3D image with the marked pixels of the two signals under consideration shown in purple (fig. 2A). From this image it is possible to determine if the signals are in different nuclei, or if a signal should be rejected because it is not in a nucleus (i.e. not coincident with the nuclear stain). However, it is not possible to determine if the signals are definitely in the same nucleus, or should definitely not be rejected, because of the absence of depth information in this image. This problem is solved by using the second image, which is a vertical 2D cut along the line joining the two signals in the 3D image (fig. 2B). From this image it is possible to determine if the signals are in the same nucleus, or if a signal should be rejected. Signals are rejected because either they are not coincident with the nuclear stain, are in a nucleus truncated by the physical sectioning of the tissue, or do not appear to be genuine FISH signals to the analyst. It is sometimes difficult to determine from this vertical cut if the nuclei are truncated, because it is possible for only a small part of the nuclei under consideration to be displayed. However, this can be solved by inspecting the third image, which is any x/y slice from the 3D image (fig. 2C). If this slice contains one or both of the marked pixels corresponding to the pair of signals under consideration, then those pixel(s) are shown in purple. Only one slice can be displayed at a time, but the displayed slice can be changed to an adjacent slice by clicking on the image, or directly changing to a slice containing a marked pixel by clicking on the left or right button in the answer menu (fig. 2D). Enumeration terminated when all signals comparisons are done, or if the "STOP ANALYSIS" button is clicked.

The efficiency of enumeration was maximized by minimizing the number of comparisons that the analyst has to make. For example, if signals "a" and "b" are determined to be in the same nucleus and similarly for signals "a" and "c", then signals "b" and "c" are not compared because they must be in the same nucleus. The number of comparisons is approximately 1.5 times the number of marked signals.

The results were displayed as a projection image prepared from the unrotated 3D image with the number of FISH signals per nucleus overlaid on the nuclei (fig. 3). The results were also stored in numerical form in files.

2.3 Software environment

The algorithms are written in C language and as an extension to the SCIL-Image software package (The University of Amsterdam, The Netherlands). The code is compiled to run on workstations equipped with the UNIX operation system.

2.4 Application of the algorithms to enumeration of FISH signals in a breast cancer specimen

A 20 μm thick section was cut from a formalin-fixed, paraffin-embedded, human breast tumor specimen. It was hybridized with a fluorescein labeled probe specific to the centromeric region of chromosome 1 and counterstained with PI as described in reference 3. The labeled section was imaged using a Leica TCS 4D confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) and imaged with a 63X, 1.4 NA objective lens. Separate green and red images, corresponding to the FISH signals and PI staining respectively, were acquired and then combined into a single color image. The image was 512 × 512 × 19 pixels in the x, y and z dimensions respectively and the pixel sizes in the x, y and z dimensions were 0.18, 0.18 and 1 μm respectively. The image was stored on the computer's hard disk. Before enumeration of FISH signals, the image was reduced to 128 × 128 × 19 pixels by mapping each 4 × 4 × 1 square of pixels in the acquired image into a single pixel. For the green component, the maximum intensity in each square was copied into the reduced image, in order to preserve the maximum pixel intensities of the FISH signals. For the red component, the minimum intensity in each square was copied into the reduced image, in order to preserve the lower intensity regions separating nuclei.

3. RESULTS

3.1 3D image display

Fig. 4 compares viewing each 2D slice of a 3D confocal image separately (fig. 4A) to viewing 2D projection images produced by

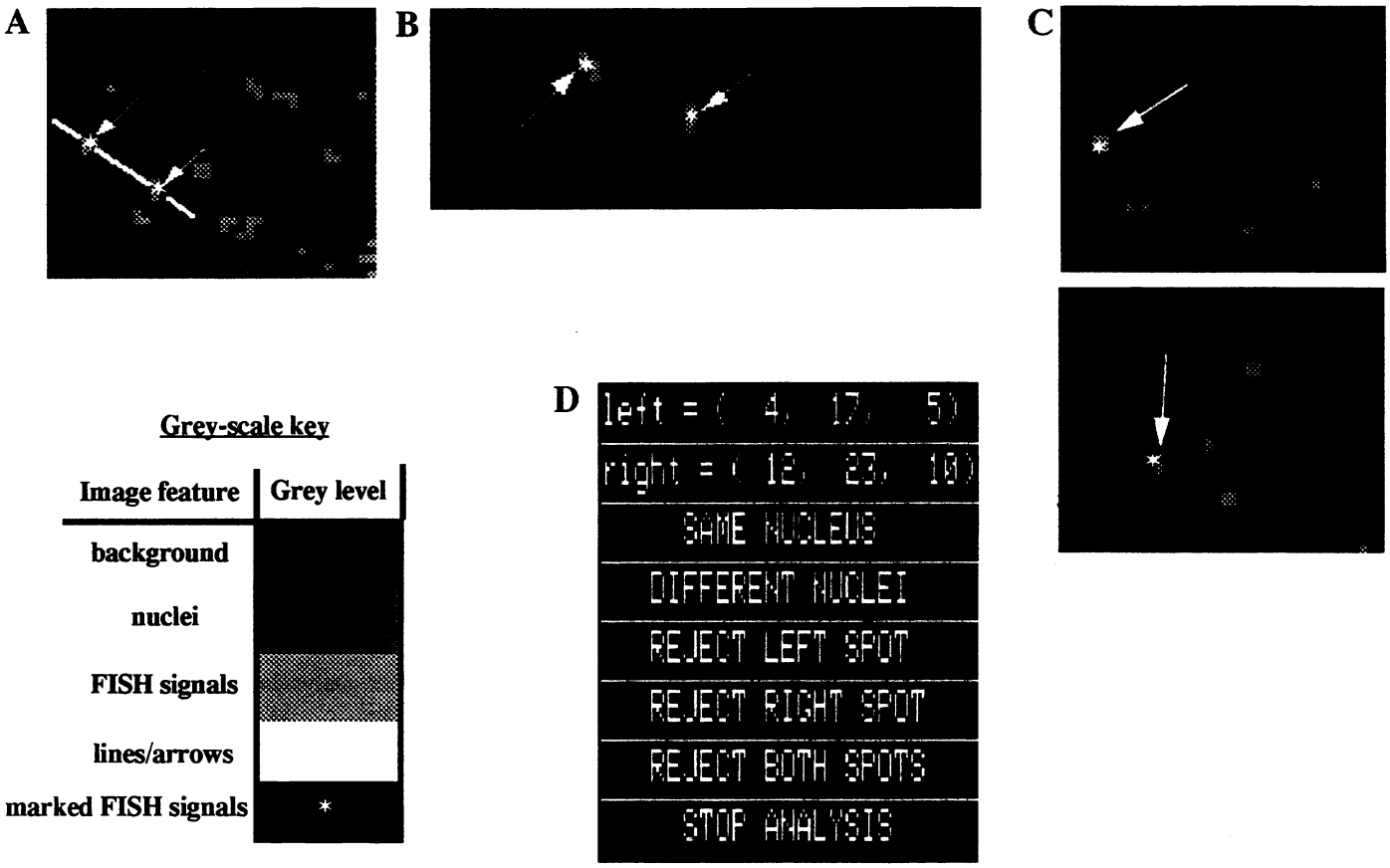

Fig. 2. Enumeration of FISH signals.

The images displayed on the computer screen are in color, however grey-scale images are shown here. In order to convert from color to grey, each color is converted to a binary image using a manually-determined threshold intensity. Pixels below the thresholds for both colors are classified as background and given a grey level of zero. Pixels above the red threshold, but below the green threshold correspond to nuclei and are given a grey level of 85. Pixels above both thresholds correspond to FISH signals and are given a grey level of 170. Lines and arrows overlaid on the images are given a level of 255. Marked pixels are shown as stars. These grey levels are shown in the key.

A: Part of a projection image prepared from an unrotated 3D image showing the PI stained nuclei, FISH signals and two marked signals under consideration (arrows).

B: Vertical slice along the white line shown in A. The arrows show the same marked pixels as A.

C: Two slices from the original 3D image each showing one of the marked signals (arrows). (In practice, only one of these slice could be displayed at a time.)

D: Menu of answer buttons. The top two buttons change the displayed slice and show the (x, y, z) coordinates of each marked pixel in the 3D image.

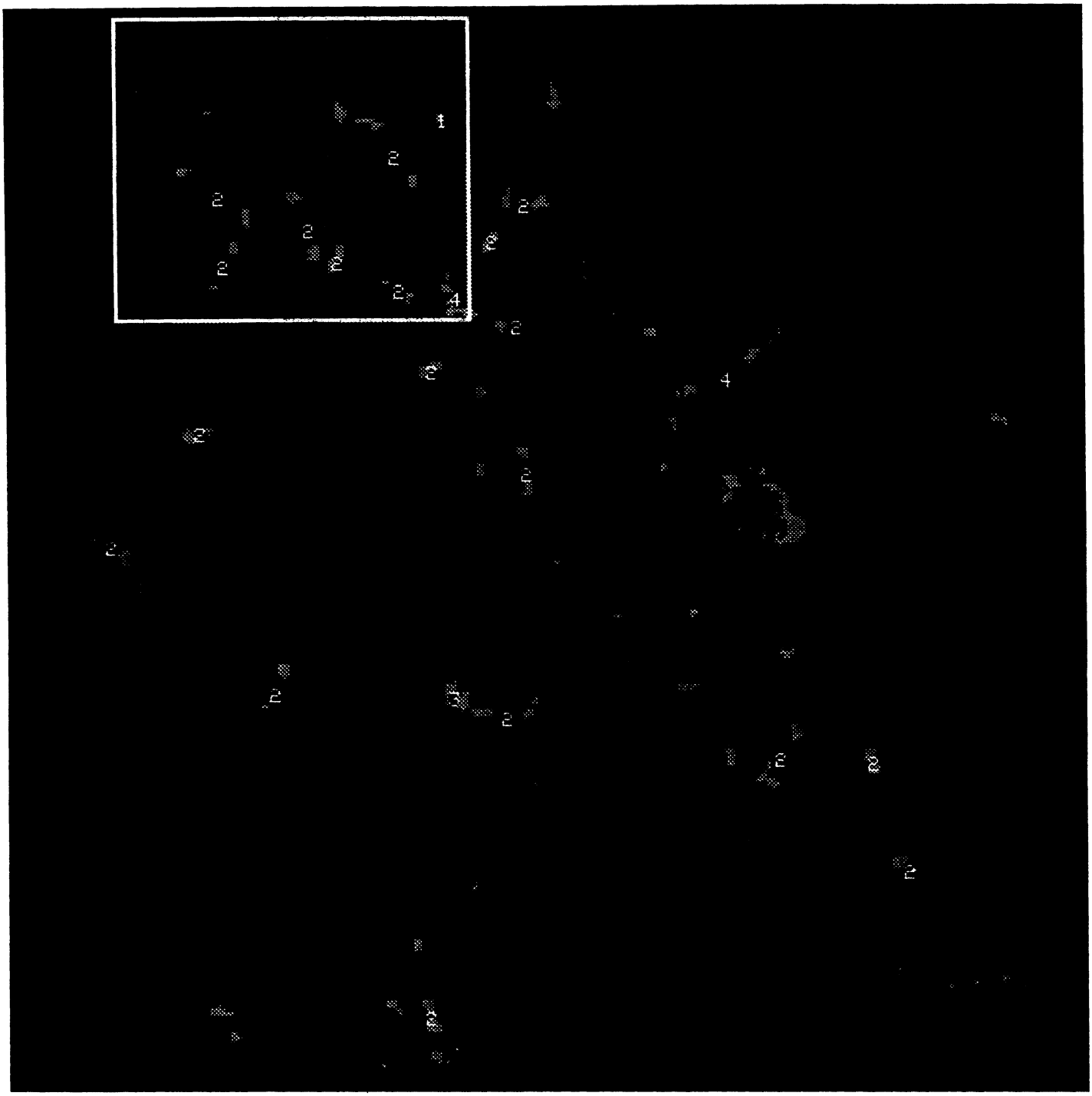

Fig. 3. The results of FISH signal enumeration in a 20 μm thick breast cancer section. The numbers are the FISH signal copy numbers in individual, intact cell nuclei. Nuclei without numbers were truncated by the physical sectioning of the tissue and are therefore not enumerated. The white square is the quadrant displayed in fig. 2A.

The color image was converted to grey using the procedure described in the caption to fig. 2.

projecting the maximum intensity pixels (fig. 4B). This projection method is more suitable for visualizing FISH signals and determining their 3D positions. This is because it preserves depth information about FISH signals in the original 3D image, by recording the z coordinate of each projected pixel in separate LUTs. This depth information is lost if the 3D image is simply collapsed into a 2D representation. Each projection image shows all high intensity signals regardless of their depth in the 3D image. The arrows in fig. 4A point to the same FISH signal, which is visible in two different slices. This makes enumeration of FISH signals difficult in these images, because multiple slices must be examined and the signals tracked. The advantage of the projection images (fig. 4B) is that all signals are simultaneously displayed in the same 2D image, but the display is busier. The only exception is when two FISH signals are directly on top of each other resulting in only the brighter signal being visible (arrow in the projection at 0° in fig. 4B). However, both signals are visible at other projection angles (arrows in the projection image at 45° in fig. 4B).

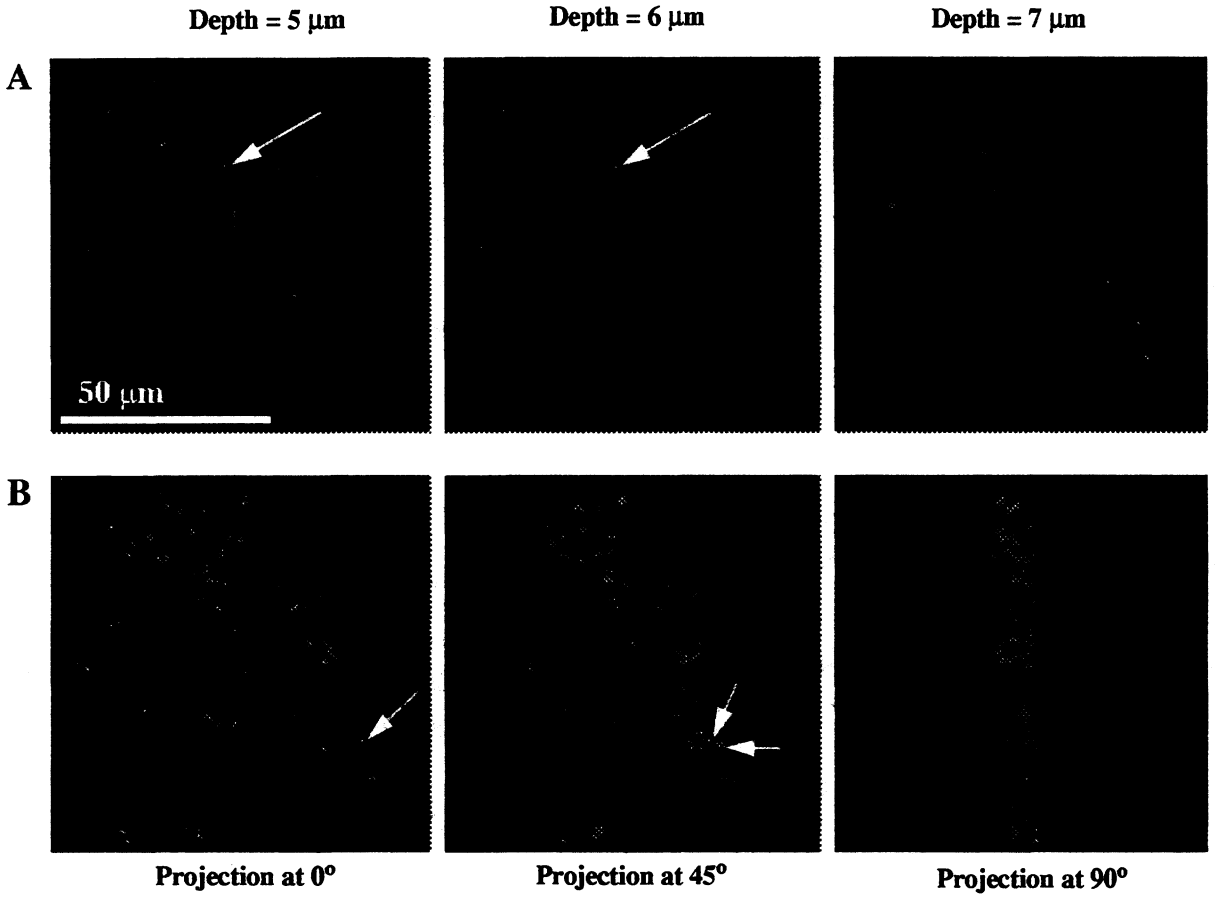

Fig. 4. Confocal images of a thick (20 μm) breast tumor section labeled with the nuclear stain, PI and a FISH probe specific for chromosome 1.

The color image was converted to grey using the procedure described in the caption to fig. 2.

A: Three 2D slices from the acquired 3D image. Enumeration of FISH signals in each nucleus is difficult using these images, because it requires careful visual examination of multiple slices for each signal. For example, the arrows in the images at depths of 5 μm and 6 μm indicate the same FISH signal.

B: Projection images through the 3D image that were generated using the 3D display algorithm. The arrow in the projection at 0° indicates two FISH signals directly on top of each other, resulting on only the brighter signal being visible. However both signals are visible at other projections (the two arrows in the projection at 45°)

3.2 Enumeration of FISH signals in a breast cancer specimen

Fig. 3 shows the results of enumerating FISH signals in a 20 μm thick breast cancer section. The number of signals in each individual, intact nucleus is overlaid on the image. Nuclei without numbers were truncated by the physical sectioning of the tissue and therefore their FISH signals were not enumerated. In this specimen, there were 23 intact nuclei and approximately 15 truncated nuclei. (In a standard 4 μm section none of the nuclei would be intact.) Of the intact nuclei, 1 had 1 signal, 19 had 2 signals, 1 had 3 signals and 2 had 4 signals. Therefore 19/23 (83%) of the nuclei had two signals.

4. DISCUSSION

We have developed two algorithms for analyzing 3D, confocal images of thick (≈ 20 μm) tissue sections. A display algorithm makes projection images through the 3D image, which has the advantage that all FISH signals and fluorescence labeled cell nuclei are visible in one 2D image regardless of their depth in the 3D image. A second algorithm enables the analyst to efficiently enumerate the number of signals in each intact nucleus. The efficiency stems from the fact that nearly all signals can be marked by a single mouse click in one 2D image. An additional advantage of the method is that it is very difficult to bias the results. This is because the analyst decides if a pair of signals are in the same or different nucleus or should be rejected without knowing the decisions that were made for previous pairs of signals. A drawback of the technique is that it can not enumerate nuclei with zero signals. However, this limitation will be solved by introducing interactive tools for directly defining the nuclear boundaries.

The technique was tested on a paraffin-embedded breast cancer specimen. It was possible to enumerate FISH signals in 60% of the nuclei indicating the potential of this technique to analyze enough nuclei to find low frequency sub-populations of aberrant nuclei.

The current algorithms represent a first step towards the development of automated enumeration. This is needed because it is expected that hundreds of nuclei per section will require analysis in order to develop an understanding of the underlying cancer biology mechanisms in the specimens. Automation has begun and has been tested on 2D images[4]. Other plans include increasing the sensitivity of the technique to allow enumeration of locus specific probes, which give small, less intense signals and measurement of the total DNA content of intact nuclei.

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6. REFERENCES

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