Technical Advance

Multicolor Deconvolution Microscopy of Thick Biological Specimens

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One limitation in understanding disease at the cellular level has been the inability to efficiently analyze DNA on a cell-to-cell basis within the natural tissue context. However, DNA analyses at a single-cell resolution should be instrumental for the understanding of cancer cell biology, cancer evolution, for chromosomal mosaic analysis and rare cell events, and should provide otherwise inaccessible information on essential biological processes. Here we present a fluorescence *in situ* hybridization-based multicolor deconvolution technique for three-dimensional microscopy. We use up to seven different color channels for probe detection, which allows the simultaneous high-resolution localization of multiple point-like sources within a biological specimen with a thickness of up to 30 μm . In addition, a DNA counterstain is used for volume labeling of the nuclei offering the opportunity for a simultaneous segmentation of nuclei. Furthermore, as the instrumentation consists of a standard fluorescence microscope it represents a low-cost method as compared to confocal microscopy. (*Am J Pathol* 2003, 162:373–379)

Despite Virchow's discovery more than 140 years ago that the single cell represents the basic unit of disease,¹ there has been a lack of appropriate tools for single-cell analysis. In particular cells within their natural tissue context, eg, within tissue sections, were deemed to be inaccessible for a detailed analysis of their genome. As a consequence, most methods, in particular the high-throughput microarray technologies for expression analyses,² are based on DNA or RNA, respectively, extracted from thousands of cells. The data obtained represent an average of all sampled cells from which DNA/RNA were

extracted. However, there is an increasing demand for efficient, cost-effective single-cell technologies for various reasons: first, it is well established that during carcinogenesis cells in the tumor tissue become increasingly heterogeneous and disorganized in their structural properties. To understand the underlying molecular mechanisms it is necessary to analyze the cells individually and within their natural tissue context. Second, the failure to achieve a more accurate prediction of disease outcome by gene expression profiling³ may be because of the heterogeneity in primary tumor populations.⁴ Thus, a sampled area may not represent the most aggressive portion of the tumor. Efficient tools for a guided dissection of specific tissue regions for a navigated RNA sampling are needed. Third, there is a growing awareness that mosaicism, ie, patches of tissue that differ genetically from the rest of their body because of a chromosomal anomaly, may contribute to common conditions such as infertility, autism, and Alzheimer's disease.^{5–8} Advanced single-cell tools should facilitate the identification of genetically different tissues. Fourth, rare cell events, eg, early pathological lesions in neoplasia or minimal residual disease should greatly benefit from the analysis of individual cells within their natural tissue context. Finally, sophisticated technologies for the simultaneous analysis of multiple signals should reveal new insights into the three-dimensional (3D)-organization of the genome within various tissues.

Current techniques for 3D microscopy generate 3D data by optical sectioning of the specimen. Up to date most 3D fluorescence microscopy is done using confocal microscopy, widely regarded as the gold standard. However, confocal microscopy has several drawbacks: the most serious drawback represents the amount of light required to produce a confocal image as the light dosage may bleach a dye. Furthermore, confocal systems that can detect a multitude of different fluorochromes using

Supported by the Bundesministerium für Bildung und Forschung (NGNF KB PO6T5), the Wilhelm Sander-Stiftung, and TILL I.D., Gräfeling, Germany (stipend to C. M.).

Accepted for publication October 14, 2002.

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the entire color spectrum from the UV to the far infrared range are high-priced and therefore not affordable for most laboratories.

Deconvolution microscopy represents an alternative.⁹ Deconvolution refers to a wide-field image restoration by computational methods used to reduce out-of-focus fluorescence in 3D microscopy images.¹⁰ Here we demonstrate a new multicolor deconvolution microscopy approach. What distinguishes our work from previous studies is that up to eight different color channels can be used for the simultaneous analysis of multiple probes. As it uses a normal epifluorescence microscope with a motorized stage it represents, compared to confocal microscopy, a low-cost alternative. The data collection is faster than for most confocals so that bleaching problems are greatly reduced. We show multicolor fluorescence *in situ* hybridization (FISH) applications, which were applied for imaging deep into specimens, such as thick (30 μ m) paraffin-embedded tissue sections. The potential of the technology is exemplified by the detection of portions within the same tumor sample with different chromosomal patterns and various degrees of chromosomal instability.

Materials and Methods

Tumor and Normal Tissue Sections

Histological sections were taken from paraffin-embedded tissue specimens, fixed in buffered formalin [4% phosphate-buffered saline (PBS)]. Normal ovarian tissue and tissue samples of two invasive ovarian carcinomas (13471B and 15078) were used. Case 13471B was a mixed mucinous/endometrioid ovarian carcinoma (grade 2, FIGO stage IA) and case 15078 was a serous ovarian carcinoma (grade 3, FIGO stage IIIC). For our analyses several serial thick sections from the same tissue block were prepared. In each case the first and last sections were stained with hematoxylin and eosin for histological analysis. In addition, 30- μ m sections of a pTa bladder cancer, fixed in 85% ethanol, were included for further validation of our technology.

Fluorochromes

Fluorochromes tested included the dyes that we use for 7-fluorochrome multiplex FISH karyotyping:¹¹ 4'-6-diamidino-2-phenylindol, counterstains DNA and was used here for the volume labeling of nuclei (excitation, 350 nm; emission, 456 nm). For DNA probe labeling we used diethylaminocoumarin (426 nm; 480 nm); fluorescein isothiocyanate (490 nm; 520 nm); Texas Red (595 nm; 615 nm); and the cyanine dyes Cy3 (554 nm; 568 nm), Cy5 (652 nm; 672 nm), Cy5.5 (682 nm; 703 nm), and Cy7 (755 nm; 778 nm). In some experiments Cy7 was replaced by LaserProIR 790 (785 nm/801 nm). Filter sets were kindly provided by Chroma Technology Corp. (Brattleboro, VT).

Table 1. Five-Color Centromere Probe Set

Probe	Chromosome	Labeling
PZ7.6B	7	Cy5.5
PZ8.4	8	Fluorescein isothiocyanate
PRB11	11	Cy5
D15Z.1/PZ17-14*	15/17*	Cy3
2Xba	18	Texas Red

*The centromere 15 probe was used for the analysis of tumor specimen 15078.

Probe Sets

Three different probe sets were used. First, we assembled a 5-centromere probe set (Table 1). These centromere probes were generously provided by Dr. M. Rocchi (University of Bari, Italy) (for detailed information see <http://www.biologia.uniba.it/rmc/index.html>).

Second, we put together a panel of seven band-specific YAC/BAC probes (Table 2) that are according to comparative genomic hybridization (CGH) data of diagnostic or prognostic value for patients with ovarian cancer.¹² This probe panel contains clones that specifically hybridize to frequently amplified genes, such as *ERBB2*¹³ or *STK15/BTAK*.¹⁴ In addition, we included probes that map in the vicinity of the *TP53* gene on 17p12 and the *MYC* gene on 8q24. The probes in both probe sets were labeled by standard nick translation.

Third, a commercially available probe set (UroVysion from Vysis, Bergisch-Gladbach, Germany) consisting of centromere probes for chromosomes 3 (Texas Red), 7 (fluorescein isothiocyanate), 17 (diethylaminocoumarin), and a probe for chromosome band 9p21 (Cy3) was used.

Tissue Pretreatment, Hybridization, and Posthybridization Washes

Paraffin was removed by xylene for 60 minutes at 55°C and ethanol for 5 minutes. After air-drying the slides were incubated in 1 mol/L of NaSCN¹⁵ for 20 minutes at 72°C and subsequently washed twice in ddH₂O for 5 minutes each. Slides were digested with pepsin [15 μ g/ml for various time periods (range, 15 minutes to 60 minutes) depending on cytoplasm and cell density] in 0.01 mol/L of HCl at 37°C. After washing with PBS, the slides were dehydrated over a graded series of ethanol (70%, 90%, and 99%) and air-dried. Before hybridization the slides were incubated in 70% formamide, 2 $\times$ standard saline

Table 2. Seven-Color YAC/BAC Probe Panel

YAC/BAC	Localization	Labeling
907g1	1p34	FITC + Cy5
773c6	1q32	Cy5
948d5	8p22	Cy3
933a5	8q24.2	FITC + Cy5.5
790c7	17p12	FITC + Cy3
230a7	17q12	FITC
3B23	20q13	Cy5.5

FITC, fluorescein isothiocyanate.

citrate (SSC) for at least 1 hour at room temperature. The slides were denatured in 70% formamide, 2× SSC, pH 7.0, at 73°C for 7 minutes.

The commercial probe kit was hybridized according to the manufacturer's instructions. The centromeric probes were dissolved in the hybridization mixture (65% formamide, 0.5% dextran sulfate, 2× SSC), denatured at 78°C for 7 minutes and hybridized on pretreated slides without preannealing for 1 or 2 nights at 37°C. An aliquot (10 μ l) of probe mixture containing labeled YAC and BAC probes (~200 ng/each), human Cot-1 DNA (10 μ g) in 50% formamide, 15% dextran sulfate, 1× SSC was denatured and applied on pretreated slides for 2 to 3 nights. After hybridization, the slides were washed three times (5 minutes each) with 4× SSC/Tween at 42°C and then three times (5 minutes each) with 1× SSC at 60°C. Blocking was done with 3% bovine serum albumin in 4× SSC/Tween for 30 minutes at 37°C. Afterward, the biotin- or digoxigenin-labeled probes were detected with avidin-Cy5.5 and sheep α -digoxigenin fluorescein isothiocyanate, respectively. The slides were then washed three times (5 minutes each) in 4× SSC/Tween at 42°C, counterstained with 4'-6-diamidino-2-phenylindol, and embedded in *p*-phenylenediamine dihydrochloride anti-fade solution.

3D Image Acquisition, Processing, and Deconvolution Technique

To obtain a 3D data set, we recorded a number of two-dimensional images of the specimen in focal planes with a 250-nm distance. A motorized Zeiss Axioplan II Imaging epifluorescence microscope equipped with an Axio-cam-MRm charge-coupled device camera was used. For image capturing and processing we used a newly developed software package. The mathematical details used in this software package were recently published.¹⁶ In brief, an accelerated maximum likelihood image restoration algorithm is used, which uses a conjugate gradient iteration scheme based on Poisson noise models. Regularization included modifications of the standard Tikhonov method, step size was determined using the Hessian matrix of the restoration functional. Here, we provide the first demonstration of the potential of the newly developed algorithms for the analysis of biological specimen. This new software tool was provided by Carl Zeiss Vision (Hallbergmoos, Germany) and has been dubbed AxioVision 3D deconvolution. A constrained, iterative deconvolution algorithm was used. Different procedures were used to select a point-spread function (PSF) as described in the text. The software package allows also the pseudo-coloring of fluorescent signals and 3D-reconstructions (AxioVision Inside4D).

The 4'-6-diamidino-2-phenylindol counterstain was used for volume labeling of the nuclei and thus for a simultaneous segmentation of nuclei. Hybridization signals were identified based on the signal intensities. Hybridization signals were assigned to segmented nuclei and counted.

Statistical Analysis

With the exception of tumor tissue 13471B, for which 64 nuclei were recorded, more than 100 nuclei/sample were evaluated. For each analyzed tissue or tissue area we calculated both the mean and the SD of the signal numbers per probe per cell. The mean values were compared with the Welch test (two-sample *t*-test assuming unequal variances), the standard deviations with the *F*-test. *P* values were subject to a two-sided significance level of 5%.

CGH Analysis

CGH was done according to previously published standard protocols.¹⁷

Results

We tested whether the co-localization and relative intensities of multiple components can be accurately studied in multiwavelength images. We were particularly interested in finding conditions to perform multicolor FISH in thick tissue sections so that hybridization signals from the intact cells in the interior portion of the section can be scored without being compromised by sectioning artifacts. Sections >20 μ m thick contain a layer of cells in the center of the section, which have not been cut.¹⁸ We decided on the use of 30- μ m-thick sections. Several experiments were designed in which single-labeled probes and combinatorially labeled probes were used.

Fluorochromes

We tested which fluorochromes are suitable for applications on thick tissue sections. The fluorochromes in the far infrared range, ie, Cy7 and LaserPro IR790, performed consistently poorly (data not shown) and were not used in further experiments. We could not achieve intensive fluorescence signals with diethylaminocoumarin in a reproducible manner on paraffin-embedded, formalin-fixed sections. However, good diethylaminocoumarin signals were achieved with a commercial probe kit on an EtOH-fixed specimen (Figure 1A), suggesting that fixation protocols may have a considerable impact on the range of applicable fluorochromes. The subsequent experiments were done with the remaining fluorochromes, ie, fluorescein isothiocyanate, Cy3, Texas Red, Cy5, and Cy5.5.

Selection of PSFs

We determined PSFs empirically and theoretically using the following information: numerical aperture of the objective, working distance of the objective, wavelength of the emitted light, XYZ dimensions of the PSF, size of a pixel, spacing between Z slices, refractive index of the immersion medium and anti-fade, and thickness of both the specimen and the coverslip. We tested different deconvolution algorithms. This was necessary as imaging at some depth into a specimen invariably introduces some

Figure 1. 3D reconstruction of nuclei within 30- μ m-thick tissue specimen after hybridization with multicolor probe sets, deconvolution, and false color assignment to the probes. The nuclei can be rotated on the computer screen and color channels can be inspected individually or in any possible combination. Note that the signals have a point-like appearance even in the optical axis. **A:** A commercially available probe set (UroVysion, Vysis) consisting of centromere probes for chromosomes 3 (red), 7 (green), and 17 (blue) and a probe for chromosome band 9p21 (yellow) was hybridized to a thick bladder cancer specimen. The reconstructed signals and the corresponding nucleus are depicted. **B:** A nucleus within a normal thick ovarian tissue specimen after hybridization with a five-fluorochrome centromere probe mix (Table 1). Two signals of each probe were detected.

spherical aberrations. Furthermore, when multiple fluorophores are used to localize different components in the same sample, the image of the distribution of at least one of the fluorophores will be spherically aberrant.¹⁹ Accordingly, for some samples an additional correction for the spherical aberration into the PSF calculation improved the results, for others, no significant difference was apparent. Examples illustrating that the selected PSFs matched the aberrations in the images very well are shown in the figures.

Five-Color Centromere Probe Set

Normal Ovarian Tissue

The five color centromeric probe set (Table 1) was hybridized to normal 30- μ m-thick ovarian tissue (Figure 1B). As expected, two signals per probe were observed in most nuclei (Figure 2A). We used the SD as a measure for the variability of the signal/probe/nucleus, which also reflects the degree of the chromosomal instability. As expected the SD was very low in the normal tissue. This low variation most likely was caused by hybridization variability and should not represent an actually existing low degree of aneuploidy within the normal tissue.

Ovarian Tumor Tissue

The centromere probe set was applied to two different ovarian tumor samples. Figure 2B illustrates the 3D-reconstruction of a nucleus from tumor specimen 13471B. In each sample the mean signal/probe/cell number was increased (Figure 2A). In addition, a tremendous variability in the signal/probe/cell number was observed, which is reflected in the increased SD. Furthermore the data suggested that regions with differences in terms of their chromosomal composition and chromosomal stability existed within the same tumor specimen. We used the cell density as a morphological criterion and compared the signal/probe/nucleus numbers in a region with mainly isolated nuclei with a region containing tightly clustered nuclei in tumor specimen 15078 (Figure 2C). We noted

surprising differences for both the chromosomal composition and the chromosomal stability. The differences in the chromosomal makeup were most obvious for chromosome 7, which was relatively overrepresented in the isolated region but relatively underrepresented in the tightly clustered area (Figure 2A). The mean signal/probe/cell numbers differed highly significantly for chromosomes 7, 8, and 18 (P values <0.008 for all three chromosomes) but were almost identical for chromosomes 11 and 15 (P values 0.87 for each chromosome).

An even more striking difference was observed for the chromosomal instability as estimated by the SD. The SD was extremely high in the isolated region (Figure 2A). In this region the signal/probe/cell numbers varied so immensely that even in neighboring nuclei completely dissimilar signal numbers were counted, which suggests that chromosomes are passed to the daughter cells in a more or less random manner. In contrast, the signal patterns were relatively constant in the nuclei of the clustered region, reflecting a relatively stable segregation of chromosomes. These differences were highly significant (P values $<10^{-3}$ for all tested chromosomes).

Seven-Color Band-Specific YAC/BAC Probe Set

We hybridized a seven-color band-specific YAC/BAC probe set (Table 2) to the tumor sample 15078. As illustrated in Figure 3, A and B, there was an exact colocalization of combinatorially labeled probes. The mean and SD of the signals were calculated (Figure 3C). As a control we corroborated the mean number of interphase signals with the CGH profile obtained with DNA extracted from the same tumor (Figure 3D). This comparison showed a close correlation between the calculated ratio profiles and the signal/cell number for six of the seven probes. The only exception was the 20q13-probe, which yielded a slightly higher mean interphase number than expected from the CGH profile. This can be explained by the considerable heterogeneity within the tumor. Taken together the results suggest that combinatorially labeled

Figure 2. A: Mean number of signals per cell obtained with a five-fluorochrome centromere probe mix (Table 1) on normal ovarian tissue (compare to Figure 1B) and on ovarian cancer specimens 13471B (**B**) and 15078. In the latter sample two separate areas (small regions within these areas are depicted in **C**), 15078 isolated and 15078 clustered were evaluated in addition. The SD reflects the observed signal variability and indicates the chromosomal instability (for details see text). **B:** Different views of a 3D-reconstructed nucleus within ovarian tumor tissue 13471B. The false color assignment corresponds to the column colors used in **A**, ie, the chromosome 7 signals are displayed in red, chromosome 8 signals in green, chromosome 11 signals in blue, chromosome 17 signals in yellow, and chromosome 18 signals in pink. **C:** Different regions based on their cell density were separately evaluated in sample 15078. Single planes of the z-stack from these two regions are depicted, the **top row** shows an area with a relative low density of cell nuclei (15078 isolated), the **bottom row** an area with tightly clustered nuclei (15078 clustered).

probes, which represent spherically aberrant images, can be restored accurately by the constrained, iterative deconvolution applied here.

Discussion

A new approach for computer-based multicolor reconstruction of individual cell nuclei is presented. Special characteristics of deconvolution microscopy may be essential for the simultaneous application of such a multitude of probes on thick tissue specimens that has not been reported before. Usually bleaching represents a serious concern for fixed specimens, which requires many focal plane images and that are labeled with sev-

eral different dyes. However, our deconvolution microscope system collects data approximately three times faster than confocals. Thus, the amount of excitation light dosage required to obtain satisfactory 3D images is so low that bleaching of the dyes is not a concern. Furthermore, the resolving power of deconvolution microscopy in Z-direction (ie, along its optical axis) is illustrated in our images. The reconstructed signals have a point-like appearance even in the optical axis suggesting that the PSFs used for deconvolution matches the aberration in the image very well. This is further exemplified by the co-localization of combinatorially labeled probes. Therefore, we conclude that a resolution as good as confocal microscopy can be achieved by deconvolution microscopy.

Figure 3. A panel of seven band-specific YAC and BAC probes (Table 2) was applied to ovarian cancer specimen 15078. **A** and **B:** Nuclei after hybridization and 3D-reconstruction. **C:** Mean number of YAC/BAC signals per cell. **D:** Corroboration between multicolor deconvolution microscopy results and CGH ratio profiles. The localization of the respective YAC/BAC clone is shown in the ratio profile, the number to the **right** indicates the mean signal per cell number as shown in **C**.

Another advantage is that deconvolution microscope systems are cheaper than confocal microscopes. However, the deconvolution approach requires computational processing, which for our samples took typically somewhere from 30 minutes to 8 hours per stack. This constraint may be greatly reduced by an efficient use of faster computers with larger RAMs.

There may be constraints on the maximum thickness of a specimen that can be analyzed. The limitation is not within imaging but rather on the FISH side as signals with a sufficient intensity have to be achieved. Thirty- μ m-thick sections represent a good compromise: they contain sufficient layers of cells that have not been cut but still allow obtaining of the hybridization signals with high-fluorescence intensities. The intensity of signals was already compromised when we tested the same protocol on 40- μ m-thick sections (our unpublished data).

Another potential limitation, which applies to all tissue analysis approaches, represents regions where nuclei are tightly clustered so that there is little evidence from the images on where the borders of the nuclei are. The use of membrane-related protein markers, such as lamins or integrins, may reduce the problem.²⁰ Currently tests are underway to test whether the combination of such fluorochrome-tagged antibodies for nuclear surface staining together with our multicolor FISH is feasible.

We observed, as expected, a very homogenous signal pattern in the normal cell population. In contrast, the

signal per cell variability was significantly higher in the tumor tissues. In tumor tissues, we observed the occurrence of regions with different aneuploid variants. Currently, we do not know which of these variants may suffer cell death or may be at selective proliferative advantage. However, this distinction may be important as it had been suggested that the predictive power of expression analysis may be compromised by this heterogeneity of tissue populations in primary tumors.⁴ Furthermore, it was hypothesized that tumors need a just-right instability to overcome new selection barriers. By contrast, if the level of genetic instability is too high, the accumulated damage in these too-high unstable cells may rise above the threshold for viability.²¹ It is tempting to speculate that cell patches as observed in the isolated area of tumor 15078 have a too-high instability pattern, which may not have any impact on the patient's prognosis but instead may hamper expression analysis by introducing significant background noise to the data. In contrast, expression analysis with RNA extracted from a just-right unstable area, which may correspond to the clustered region, may improve the predictive power of expression analysis considerably.

Only a technology as presented here may contribute to the identification of areas with different chromosomal and instability patterns and opens avenues for new strategies for an improved resolution of expression data. A combination of interphase cytogenetics and gene expression

analysis can be achieved by cutting serial sections from a tissue specimen. One section is used for FISH. The fluorescence signals can then be used as a template to guide dissection from the corresponding parallel tissue sections. After RNA isolation and microarray gene expression analysis, the results can, if desired, be interpreted again at single-cell resolution by application of one of the latest multilabeled mRNA *in situ* hybridization detection methods on further serial sections.^{22,23} Further potential applications for our new single-cell analysis approach include the elucidation of the spatial order of the genome in differentiated cell tissue and the analysis of pathological processes in atypical proliferation, metaplasia, preneoplastic lesions, and carcinoma *in situ*.

Acknowledgments

We thank Dr. S. Wagenpfeil for advice in statistical analysis; Drs. M. Cremer, T. Cremer, and R. Knuechel for critically reading of the manuscript and helpful discussion; and Margit Müller for expert technical assistance.

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