Comparative Genomic Hybridization Imaging by the One-Chip True-Color CCD Camera Kappa CF 15 MC

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In comparative genomic hybridization (CGH), quantitative measurement of fluorescence intensity ratios on metaphase chromosomes is the basic method for detecting copy number changes in the test DNA. The microscope images are usually acquired by high-resolution, highly sensitive, black and white (B&W) CCD cameras. This requires subsequent recording of the different color images using appropriate filter combinations for excitation and emission. We describe an alternative approach using the one-chip true-color CCD camera Kappa CF 15 MC and an Omega triple-bandpass filter for simultaneous registration of the three dyes Texas red, FITC, and DAPI. A detailed examination of the imaging properties of the System was performed. The camera response in the three color planes R, G, and B was evaluated, and calibration factors for image correction were calculated. An error estimate is given.

A complete computer program for the image analysis of CGH experiments has been developed for an 80486 PC, using the commercially available software package Optimas as the basis for image recording. Examples that confirm the suitability of the System for ratio imaging in CGH are presented. The results were compared with others obtained from the same microscope slides using an established setup consisting of a B&W CCD camera (Photometrics) and a Software program based on the TCL Software package and run on a Macintosh Quadra 950. The results obtained using die two different Systems were found to correspond closely.

Key terms: One-chip true-color CCD camera, quantitative fluorescence microscopy, ratio imaging, comparative genomic hybridization

In cytogenetics, fluorescence microscopy is about to become one of the most important tools for quantitative analysis. A highly specific technique to delineate chromosomes, chromosomal subregions, or individual genes is fluorescence in situ hybridization (FISH; 6, 7, 11, 16, 17). Meanwhile, it has become possible to label different chromosome regions with different fluorochromes simultaneously (22). A very promising application of the FISH technique is comparative genomic hybridization (CGH; 8, 14, 15, 23, 25), which offers an important tool in tumor diagnostics. For this approach, after preannealing with blocking DNA (e.g., Cot-1 DNA), differentially labeled tumor and normal genomic control DNA are hybridized together to metaphase spreads obtained from normal cells. Different affinity molecules are coupled to both DNAs. After detection of hybridized sequences with two different fluorochromes, the respective fluorescence intensities are measured along the chromosome axes. Because a defined ratio of DNA concentrations is used in the hybridization, the normalized ratio between the fluorescence intensities reflects the relative copy number of chromosomal segments in the tumor DNA. Two fluorescence colors (e.g., green FITC and red Texas red signals) are needed for ratio imaging. A third fluorochrome that is not bound by in situ hybridization via DNA probes (e.g., blue DAPI signals) may be required for segmentation and simultaneous banding analysis in order to identify the chromosomes.

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Depending on the goal of the investigation, a combination of cooled or noncooled CCD cameras of appropriate sensitivity and resolution, along with Computer hardware and software for image analysis, is used for the quantitative microscopy of FISH Signals (4, 7, 19, 24, 27). The requirements for CGH analysis are still a point of scientific discussion among instrument engineers and appliers. For clinical use, the challenge is to develop sophisticated but cost-effective CGH analysis Systems (21).

Regarding the image acquisition hardware (detection optics, camera), the present consensus (9) favors standard epifluorescence microscopes with high-resolution, highly sensitive, low-noise and unfortunately highly expensive black and white (B&W) CCD cameras. However, it has been recognized that simpler cameras may be sufficient for many CGH applications. Here we introduce examples of CGH analysis using the less expensive, cooled, true-color CCD camera Kappa CF 15 MC and a microscopy image analysis System on a general-purpose 80486 PC.

In contrast to B&W cameras, with which each color requires the acquisition of one separate image with an appropriate filter combination, a color camera makes it possible to obtain the complete information in one image. Fluorochrome excitation and fluorescence emission must therefore be performed via triple-bandpass filters sufficiently adapted to the spectral properties of the fluorochrome combinations being used. This, however, does not overcome the problem of cross-talk between the three color image planes red, green, and blue (RGB).

Cross-talk arises owing to the fact that a color camera such as the Kappa CF 15 MC does not register the Signal intensity of one fluorochrome in one color plane only.

Instead, a mixture of signals is registered that depends on the overlap of the fluorochrome spectra, the band shape and size of the filter used, and the spectral response of the mosaic filters on the camera chip. Typically, four neighboring pixels of the chip carrying four different filters (cyan, yellow, green, magenta) provide signals that result in three color values for one point in the RGB image. These shortcomings can be compensated for through image-analysis software (2, 3). Therefore, the signals of each fluorochrome (Texas red, FITC, DAPI) used in the experiment were calibrated separately. From these calibrations, it is possible to deduce the pure color values in RGB of up to three fluorochromes and to correct the CGH images.

**MATERIALS AND METHODS**

**The Microscope**

A fluorescence microscope (Leitz Orthoplan; Leica, Wetzlar, Germany) equipped with a 50 W mercury arc lamp was used, to which the cooled, color CCD camera CF 15 MC (Kappa, Gleichen, Germany) was mounted. By means of a triple-bandpass filter (XF 56; Omega Optical, Brattleboro, VT; for excitation and emission spectra, see Fig. 1), it was possible to excite the dyes and to record the fluorescence signals of three different fluorochromes simultaneously. In this report, fluorescein isothiocyanate (FITC; emission at 495-575 nm), sulforhodamine 101 (Texas red; emission at 580-660 nm), and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; emission at 405-540 nm) were used. An oil Immersion objective lens with a numerical aperture of 1.3 and a magnification of x63 was used to acquire the images. Furthermore, a tubus magnification lens (x 1.25) was used.

**The Kappa CF 15 MC Camera**

The Kappa CF 15 MC camera works with a cooled interline CCD chip with integrated mosaic filter and in interlace mode. The CCD chip consists of 681 (H) x 582 (V) pixels. By means of a multicontrol unit (MCU), it is possible to adjust the R and B gain relative to the signal. The frame grabber of the camera provides 8-bit signal resolution for each color plane; therefore, relative signal values between 0 and 255 are possible. The signal-to-noise ratio of 44 dB (data given by manufacturer) is comparable to that of currently used B&W cameras.

The four-color mosaic filter provides a higher spatial resolution in all color planes than earlier designs, which gave sufficient resolution only in the medium spectral range of visible light. The
FIG. 2. a: The four-color mosaic filter of the Kappa CF 15 MC camera. The example concentrates on the first half-image. For line n, the green and yellow pixels (region I) as well as magenta and cyan pixels (region II) are added up. This results in different representation of color information. Addition of regions I and II gives a luminance signal, whereas subtraction yields a chrominance signal. Für line (n + 1), the combination of color filters changes: Green and cyan are combined to obtain the region I color information; magenta and yellow yield the region II signals. Adding the regions gives the luminance signal again, but subtraction gives a second chrominance Signal. In the second half-image, the roles of line n and line (n + 1) are reversed. b: Fourier modulation transfer function (FMTF) of the green color channel. The resolution was tested by means of a resolution test target (Ealing Corporation), which consists of groups of parallel lines. A group of 15 parallel lines spaced 2 μm apart was projected onto the CCD chip by an objective lens (x10, NA. 0.45). The vertical distance between two lines was then 2.3 pixels, equivalent to an expected resolution of 440 of 512 pixels. The intensity profile along these distances (i.e., perpendicular to the lines) was measured. The plot shows the normalized intensity of the FMTF (sample size 32 pixels) vs. frequency in units of the Nyquist frequency.

signals in two vertically adjacent mosaic filter pixels are added before digitization. They are combined with their horizontal neighbors by addition or subtraction, giving a luminance signal and a chrominance signal. Two half-images are taken, in which the vertically adjacent mosaic filter pixels are combined in two different ways (see Fig. 2a). The luminance signal resolution is twice as good as the resolution achieved for each chrominance signal. Before digitization, the luminance/chrominance signals are changed electronically into RGB Signals. The two half-images are digitized independently into the 512x512 pixel frame buffer. This means, although four mosaic filter pixels (2x2) describe the color value, the resolution is not decreased by a factor of 2 compared with the pixel numbers but by factors of only approximately 1.5 (H) and 1.33 (V), respectively, owing to the recording of two half-images. An optical low-frequency bandpass filter (Sony) placed in front of the camera prevents aliasing effects and ensures a resolution that is similar in all three color planes to a great extent.

As a result of the requirements of simultaneous color registration, the spatial resolution of the Kappa CF 15 MC camera is lower than that of B&W cameras with comparable pixel size. However, the resolution proved to be sufficient for many purposes of quantitative microscopic image analysis of fluorescence-hybridized chromosomes (4, 7, 12). The Kappa CF 15 MC achieves a resolution of 460 (H) x 440 (V) pixels out of 512x512 pixels (information given by the manufacturer). The absolute magnification was calibrated by means of a high-resolution test target (Ealing Corporation). One image pixel corresponds to 156 ± 5 nm horizontally and 106 ± 4 nm vertically. Taking the Nyquist theorem into consideration, structures of a size of

\[ D_{\text{min, horiz.}} \approx (256 \pm 5) \text{nm} \times (512/460)^2 = (347 \pm 11) \text{nm} \]

horizontally and

\[ D_{\text{min, vert.}} \approx (106 \pm 4) \text{nm} \times (512/440)^2 = (247 \pm 9) \text{nm} \]

vertically can be resolved. This fits the objective resolution given by

\[ D = (0.61/\text{N.A.}) \times \lambda \]

for most emission wavelengths of the dye complexes usually applied for FISH. For instance, the filter emission band for FITC is centered around 530 nm. With numerical aperture (NA) = 1.3, this leads to a minimal
distance of \( D \sim 249 \text{ nm} \) that can be spatially resolved. However, the sampling theory that leads to the Nyquist criterion is based on sampling with delta pulses. The situation for a CCD camera is much more complex: Even for B&W cameras, the resolution is not given by the absolute horizontal and vertical pixel numbers. The digitized image results from digitization of the video signal provided by the camera, and this determines the resolution. Techniques to determine the camera resolution under different conditions were described by Auer (1). By following the principles of these considerations, the resolution as stated by the manufacturer was confirmed. As an example, Figure 2b shows a Fourier modulation transfer function for the green channel. A compatible result was obtained for the red channel.

The CGH Analysis Software

To obtain the fluorescence ratio profile that maps relative chromosomal changes in CGH, an analysis program was developed within Optimas, a commercially available software package that runs on an 80486 PC under Microsoft Windows. First, an image was smoothed with a 3x3 binomial filter (13) in order to reduce noise and to obtain the same resolution for all color planes. It was then scanned for the smallest fluorescence signal value in each color plane, and this was subtracted from the original image.

The individual fluorochrome signals were then calculated using the results of the calibration (see next section) and the solution of the equation system given in Table 1. The background was estimated by applying a three-step Gaussian pyramid to an image, a process similar to applying a low-pass filter but more time efficient (13). This process yielded a smaller matrix containing only higher structures and neglecting local intensity variations. The background was defined as the smallest value in this matrix and was subtracted from the image.

The intensities in the color plane containing test DNA signals (green) were normalized relative to the reference DNA signals (red) using a method similar to that described by Lundsteen et al. (18): The image pixels connected to foreground were found by thresholding. The histograms of the red and green channels were calculated for these foreground regions. The ratio between the most frequent intensity level of each histogram was taken as a normalization factor; this intensity level should correspond to a balanced state of fluorescence ratio.

Segmentation was carried out using an algorithm that combines region growing methods and multiresolutional image processing. It has been described by Cibulskis and Dyer (5). The segmentation image was provided by averaging the blue and green color planes. After segmentation, a two-step erosion process was
performed. Lines were created along the chromosomal axes, starting from the center of mass and moving along the arms of the chromosomes. The lines were extended by two pixels beyond the edges of the boundaries of the segmentation mask to even out the erosion effect. If lines were not placed correctly by the computer, the user replaced them by hand. The chromosomes had to be identified by the user. The lines were subdivided into segments, the number of segments depending on the number of the chromosome (e.g., 100 segments for chromosome 1). The fluorescence of each segment was obtained by sampling the green and red pixel values perpendicular to the chromosomal axis. Then, the values of these pixels were averaged for green and red, and the ratio of these averages was computed. This method of obtaining the ratio profiles is analogous to the method used by du Manoir et al. (10).

After all metaphase spreads were processed, a final profile was determined by averaging all fluorescence ratios obtained for each segment in each chromosome. The approximate 95% confidence interval of the estimated mean as defined by Piper et al. (20) was also calculated.

Calibration of the Fluorochrome Signals

After image acquisition, three values for the color intensities at each image point are read out from the camera. These values do not contain specific information regarding the intensities of the individual fluorochromes. The green value (G) of an image pixel, for example, contains signals from DAPI, FITC, and Texas red, and the situation is similar for the R and B color plane (see below). Nevertheless, because the camera response to light intensity is linear (see Fig. 3), it is possible to deduce the individual signals of three fluorochromes using the information from the RGB Signal. The relationship between the color plane intensities has to be known for each separate fluorochrome. From the intensity of a fluorochrome in one color plane plotted as a function of its intensity in another color plane, a calibration curve was obtained. These curves were extracted from single-color hybridizations with the same fluorochromes that were used in a three-color experiment. Owing to the camera's linear response, the ratio of one color plane intensity to another is constant, so the calibration curves that emerge are straight lines.

From the hybridization of a painting probe labeled with Texas red to which no counterstaining was added, the ratio of the R to G Signal at an image pixel, as well as the B to R ratio, was measured for several metaphase spreads, and similarly for FITC and DAPI. The measured intensity of a fluorochrome was taken as the abscissa value (i.e., G for FITC, R for Texas red, and B for DAPI), and the ordinate was used for the second color value. The calibration led to a straight line with only two parameters for the gradient and the segment (Figs. 4-6).

In the experiment, the standard adjustment on the MCU was used; i.e., red, blue, and gain
were set to the middle values, and the offset was set to a small value (five digits out of a maximum number of 32). This adjustment was not changed during the entire experiment. With these MCU settings, FITC did not feature in the R or B plane.

The following equation system was obtained for FITC, Texas red, and DAPI:

\[
\begin{align*}
\text{R}_{\text{Tex. Red}} + \text{r}_{\text{DAPI}} &= \text{R} \\
\text{G}_{\text{FITC}} + \text{g}_{\text{Tex. Red}} + \text{g}_{\text{DAPI}} &= \text{G} \\
\text{B}_{\text{Tex. Red}} + \text{b}_{\text{DAPI}} &= \text{B}
\end{align*}
\]

where R, G, and B are the registered signal values, whereas r, g, and b stand for the contributions of the different fluorochromes. This results in four calibration lines:

\[
\begin{align*}
\text{r}_{\text{DAPI}} &= a_1 \cdot \text{b}_{\text{DAPI}} + c_1 \\
\text{g}_{\text{DAPI}} &= a_2 \cdot \text{b}_{\text{DAPI}} + c_2 \\
\text{g}_{\text{Tex. Red}} &= a_3 \cdot \text{r}_{\text{Tex. Red}} + c_3 \\
\text{b}_{\text{Tex. Red}} &= a_4 \cdot \text{r}_{\text{Tex. Red}} + c_4
\end{align*}
\]

This system of linear equation can be expressed in matrix algebra:

\[
\begin{bmatrix}
0 & 1 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 1 & -a_1 \\
0 & 0 & 0 & 1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & -a_3 & 1 & 0 & 0 & 0 & 0 & -a_4 & 0 & 1 & 0 & 0 & 0
\end{bmatrix}
\begin{bmatrix}
\text{r}_{\text{FITC}} \\
\text{g}_{\text{FITC}} \\
\text{b}_{\text{FITC}} \\
\text{r}_{\text{DAPI}} \\
\text{g}_{\text{DAPI}} \\
\text{b}_{\text{DAPI}}
\end{bmatrix} =
\begin{bmatrix}
\text{R} \\
\text{G} \\
\text{B}
\end{bmatrix}
\]

There is a definite solution if the determinant of the matrix is non-zero. It was calculated to be \(-1 + a_1 \cdot a_4\).

The solution of this system is given in Table 1. This procedure is different from that described by Castleman (2,3). Here, the contribution of one dye to each color plane can be determined separately, with the advantage that the system response can be taken into account. For other dye combinations, the calibration can be performed in the same way. This was tested for DAPI, FITC, and CY3 (data not shown).

![Graph showing hybridization of genomic DNA detected with avidin-Texas red.](image)

**Fig. 4.** Hybridization of genomic DNA detected with avidin-Texas red. The exposure time was set to 32 s. The abscissa shows the signal value in the red color plane. The ordinate shows the average signal value in the green or blue color plane, respectively, at the pixel with the red value given by the abscissa. The error margins are given by the standard deviation of the averaging process. To obtain the average value in the green color plane at a red value of 50, for example, all pixels in the image with a red value of 50 were recorded. For the same pixels, all green values were recorded, and their average and standard deviation were calculated. This was done for all red values present in the image. The data fits to obtain the straight lines are based on a least squares method, with each data point weighted with its error margin.
One-Color Hybridizations for Calibration and CGH

For the CGH experiment, DNA was extracted from cells of a testicular germ cell tumor (seminoma) and amplified by DOP-PCR (25,26). CGH was carried out as described previously (8), with minor modifications.

Briefly, 300 ng of biotinylated tumor DNA was mixed with the same amount of digoxigenin-labeled control DNA from a healthy male and hybridized to reference metaphase spreads (46, XY) under suppression conditions. Biotinylated tumor DNA sequences were visualized by Texas red-conjugated avidin, whereas digoxigenin-labeled control DNA was detected by indirect immunofluorescence using mouse antidigoxin and sheep antimouse Ig-FITC antibodies. Chromosome preparations were counterstained with DAPI. A detailed cytogenetic description of the tumor material used has been published elsewhere (26).

For calibration measurements, two single hybridizations following the same protocol were performed. Biotinylated DNA from a healthy male was visualized by Texas red as described above, and, in a second experiment, digoxigenin-labeled control DNA was visualized by FITC. For the DAPI calibrations, normal metaphase spreads (46, XY) were counterstained.

CGH Analysis by a Standard Method

To test the results obtained for ratio imaging by means of the algorithm described above, the CGH experiment was analyzed using a procedure that is well established and has been described in detail previously (23, 25). Individual color images were recorded subsequently via single-bandpass filters with an epifluorescence microscope (Zeiss Axiophot) to which a cooled B&W CCD camera (Photometrics, Tucson, AZ) was mounted. A Plan Neofluar objective (x 63, NA 1.25) was used that provides a spatial resolution of approximately 259 nm for FITC according to the criterion mentioned above. The 12-bit images obtained from the camera were converted into an 8-bit format for further analysis. After image matching, calculation of the fluorescence ratios was performed with a CGH evaluation program, as described elsewhere (10). This program is based on a TCL Software package that runs on a Macintosh Quadra 950.
RESULTS

The results of the calibration measurements for DAPI, FITC, and Texas red are shown in Table 2. On the basis of these results, the determinant was evaluated to be \( \det(A) = -0.9855 \pm 0.0017 \). The error margins, i.e., the average errors obtained with the Gaussian error progression method, of the algorithm for obtaining the pure fluorochrome signals were also calculated. To estimate the error of the fluorescence ratio, the relative error margins of each color plane are important:

\[
\Delta \left( \frac{\text{color } A}{\text{color } B} \right) = \frac{\Delta \left( \frac{\text{color } A}{\text{color } B} \right)^2}{\left( \frac{\Delta \text{color } A}{\text{color } A} \right)^2 + \left( \frac{\Delta \text{color } B}{\text{color } B} \right)^2}
\]

(for example: A = red, B = green).

The graphs that are presented therefore show relative error margins. For FITC, the standard deviation depended on the value of its own signal, as well as the signals of the other two fluorochromes. For DAPI and Texas red, it depended only on their own and each other’s signals, since FITC signals did not appear in the R and B color planes. A three-dimensional (3D) plot was chosen to show the dependence of the error margin percentage on the signal values (Figs. 7-9). For FITC, two plots are presented; one shows the dependence of the error margin on the DAPI signal, and the other shows its dependence on the Texas red signal. It can be seen that the DAPI signal has a greater influence on the G color plane than has Texas red (Fig. 8).

The Texas red error margins stay below 6% if the evaluated signal value is greater than 50 units (out of a possible 255, as explained above). Exposure times are usually chosen to separate interesting objects from background, so this condition can easily be fulfilled. The situation is similar for FITC. Here, the standard deviations are slightly higher but, as is shown in Figure 8, remain below 12% if the FITC signal is greater than 50 units and the DAPI signal does not exceed 170 units. In this experiment, this was true in chromosome areas because the triple bandpass filter did not yield a very high throughput for DAPI fluorescence. The standard deviation of the evaluated DAPI signal shows only a weak dependence on the Texas red signal. It is within a region of 3-6%, except for the case of low DAPI and high Texas red signal,
but it does not exceed 8% if the DAPI signal is greater than 50 units.

The algorithm was applied to evaluate metaphase spreads for the CGH experiment described in Materials and Methods. The ratio profiles that emerged after image acquisition with the Kappa CF 15 MC camera, and calculation of the corrected values as explained above, were compared with those resulting from the standard method. This calculation required approximately 30s for a typical image size of approximately 500 x 400 pixels. To compensate for unequal sensitivity and efficiency for the green and red fluorochrome, these two color channels were normalized as described by Lundsteen et al. (18) before the ratios were calculated.

The DAPI banding pattern visible in the blue color plane was not suitable for identifying the chromosomes. This was due mainly to the low intensity of the excitation light (Hg; 50 W); the excitation band of the filter for DAPI did not correspond to the intense spectral lines of the lamp, nor to an increased noise level in the blue channel of the camera, which did not show up in the red or green channel. The blue image was therefore used only for calculation of the chromosome segmentation mask. To identify the chromosomes, a separate DAPI image was recorded using a single-bandpass filter.

Figure 10a shows the ratio profile after analysis of six metaphase spreads with the standard B&W method, and the results obtained with the color method are shown in Figure 10b. The three vertical lines define the expected fluorescence ratios for losses, balanced states, and gains. The left line defines a threshold at a ratio of 0.75. If the ratio exceeds this lower threshold, a deletion was present in more than 50% of the cells used for DNA extraction. The upper threshold at 1.25 defines the same condition for amplification. This example was included to show the suitability of the color method. It is summarized in Table 3.A detailed analysis of genetic changes in the tumor has been published elsewhere (26; in this study, TRITC was used instead of Texas red).

The standard method shows definite gains on 12p and 8pter-q21.3. Chromosomes 19, 20, and 22 are overrepresented, as is 21q22. The gains on these small chromosomes are artefacts introduced during DNA preparation. Nevertheless, these gain regions remain useful for comparison of the two methods. The upper threshold is also exceeded on 1q21-q25, 1q42-qter, 2q31-qter, 9q31-qter, 10q26, 12q24, 16p, and 17q. On chromosomes 1 and 14, the gains in regions near the centromeres are most likely due to a relatively high affinity of Texas red-labeled probe for these suppression regions.

FIG. 7. Error margins of the calculation of the individual Texas red signals. The standard deviation of the evaluated Texas red signal value is given as a percentage. It was obtained by the Gaussian error progression method, where the errors of all parameters (Table 1) are taken into account. It does not depend on the FITC value, however; this value is not needed to calculate the Texas red signal. The x axis shows the individual Texas red signal, the y axis the individual DAPI signal. The z axis then gives the percentage error margin of the calculated value for Texas red. The error margin stays below 6% of the evaluated signal if the Texas red signal exceeds 50. If the Texas red signal value equals 60 and the DAPI signal equals 250, for example, the plot yields 6%, in other words \( \text{VALUE}_{\text{Texas Red}} = 60 \pm 3.6 \). The sawtooth pattern is due to the changes in absolute values of the error margins (due to digitization) as the Texas red signal increases: When the absolute error jumps from one to two units, the relative error increases suddenly (e.g., from 1% to 2% as the Texas red signal rises from 100 to 105, with the DAPI signal at a constant 40).
The same microscope slide was used for the color camera method; 9 metaphase spreads were analyzed. The gains and losses shown in Figure 10b correlate closely with the results obtained with the standard method. Chromosomes 12p, 19, 20, and 22 are also clearly overrepresented. The upper threshold is crossed as well at 1q21-q22, 8p-8q21.1, 10p, and 16p. Chromosome 10 emerges as being on the border of the upper threshold in both analyses, whereas 11q22 almost reaches the border of the lower threshold in both Figure 10a and Figure 10b. The X chromosome ratio shows raised values but does not exceed 1.5, which would be the upper threshold in this case, in so far as both tumor and reference DNA were extracted from (XY) cells.

The overrepresentation sites on 1q42, 2q, 9q, 12q, and 17q that emerged with the standard method were not detected by the color camera method, but the ratios of these regions come close to the upper threshold, which was defined arbitrarily. A gain on 2q32-qter is possible judging from the results of the color camera method, because the confidence interval crosses the threshold.

DISCUSSION

CGH has become a challenging tool in tumor cytogenetics. The microscopic analysis of CGH specimens is based on the quantitative ratio measurement of fluorescence intensities. Several laboratories have developed software packages for CGH image analysis and evaluation (10,18,20). For image acquisition, all these laboratories use B&W CCD cameras. These cameras provide a high linearity, and the exposure tunes can easily be adapted to the fluorochrome intensities. Systems with a configuration similar to that of the B&W system used in this study can be regarded as standard systems for CGH. The B&W CCD camera commonly used (e.g., Photometrics) provides a high spatial resolution, high sensitivity, low noise, and a high dynamic range (e.g., 12 bits). Hardware and software requirements for CGH imaging have been defined recently (9). It was suggested that a simpler version of the CCD camera could probably be used. Here we have shown and tested an alternative method of CGH imaging using the one-chip, true-color CCD camera Kappa CF 15 MC. The CGH Images were analyzed by a homemade software package with algorithms based on the ones established for B&W Systems. The Software was implemented on a general-purpose 80486 PC running Microsoft Windows and Optimas. In comparison with most established systems for CGH analysis using high-resolution B&W cameras, the Kappa system is a very cost-effective one, an argument that may be especially significant for routine applications (21). Apart from the costs, however, the performance of a system also has to be proved by the results attained in CGH analysis and considering the advantages or disadvantages in handling. A few examples were therefore compared using a B&W system as a standard, which has meanwhile been applied in several laboratories (8-10,25,26). Although it is not the aim of this article to present a detailed cytogenetic interpretation of the cell type used, both systems detected typical aberrations, the two sets of results showing a strong correlation.
FIG. 9. Error margins of the DAPI signal evaluation. The standard deviation of the evaluated DAPI signal value is given as a percentage. It does not depend on the FITC signal and remains roughly constant over a wide range of DAPI and Texas red values.

These results were also compatible with those of Speicher et al. (26). As far as the differences are concerned, it should be remembered that the thresholds for the decision of over- or underrepresentation of DNA are relatively arbitrary. Furthermore, the crossing of such a threshold depends on the statistics (number of cells analyzed), the evaluation program (e.g., smoothing operations), and the reproducibility of the FISH procedure from cell to cell. Intra- and intercellular variations of the fluorescence signals of approximately 15-30% have been reported for repetitive probes (4,12,19). It is anticipated that similar results might be obtained for genomic probes. There may also be differences depending on the fluorochromes used. This can be seen by comparing the results of Speicher et al. (26), who used TRITC, and the results shown here, obtained with Texas red staining. The same standard image analysis system was applied in both cases. It may be useful to include an error estimate (e.g., confidence ranges) in the final result of the CGH analysis (see Fig. L10b). A comparison of various sophisticated analysis systems and procedures may be made easier by taking an appropriate error estimate as a basis.

One argument against the application of the Kappa CF 15 MC camera for CGH imaging is the low spatial resolution of color cameras, especially in the direction where the response of neighboring pixels is mixed to obtain the color information. In the case of long time exposure (>20 ms), this disadvantage can be reduced by acquiring two half-images. Our results indicate that, under this condition, the resolution of the camera is close to the theoretical resolution of a high-numerical-aperture lens for FITC fluorescence and other dyes of higher fluorescence wavelengths. The development of digital CCD cameras (e.g., Kappa CF 20 DXC) may be a promising tool to increase further the resolution of one-chip true-color cameras.

B&W cameras require the acquisition of one separate image for each color (= 3 images for CGH) via different optical filters or filter combinations. A possible image shift resulting from different optical pathways has to be considered in image matching. If the sample is sufficiently bright, this problem can be avoided by using a triple-bandpass filter for emission and, in addition, placing separate bandpass filters for each labeling color in the optical pathway of the excitation beam. It is still necessary to acquire three separate images for CGH, however. If a color camera is used, a triple-bandpass filter is necessary. With its multibandpass excitation, the complete color information of the image can be recorded in one acquisition step. Because of the lower light sensitivity of the Kappa color camera, the acquisition time for one image was on the order of 30s. In practice this was no longer than the typical acquisition time for three images with the B&W camera (5-14 s each), allowing for manual filter change.

Multibandpass filters produce lower transmission and higher cross-talks than single-bandpass filters. Also, multibandpass filters are not yet available for all dye combinations and all types of microscopes. Whereas more than three dyes can be separated by individual filters using a B&W camera, the color camera is still limited to three dyes at a maximum, i.e., the application of a triple-band-pass filter. These are indeed basic problems in the application of color cameras. Further development of dye complexes and sensitive multicolor approaches in FISH requires new filters of higher transmission. We thus expect that more multibandpass filters will be designed in the future.

It should be mentioned that, in the example described here, an additional DAPI image was

FIG. 10. Example of CGH profiles obtained by the two methods. The ratio exceeding the lower/upper threshold corresponds to a loss/gain in more than 90% of the cells used for DNA extraction (10). a: The standard method reveals definite gains on chromosomes 8pter-q21.1, 12p, 19, 20, 21q22, and 22. b: The color camera method shows the average ratio (dark line) and the 95% confidence interval (indicated by the light lines). Chromosomes 8pter-q21.1, 12p, 19, 20, and 22 show definite gains. For further discussion, see text.
registered to facilitate chromosome identification according to the banding pattern. This appears not to be a basic problem of the color camera system but a problem of the Omega XF56 filter in combination with the brightness of DAPI for banding. Because color cameras do not allow optimization of the gain settings for each separate color, this shortcoming can be overcome only by using another triple-bandpass filter with higher transmission for DAPI fluorescence.

Because the RGB signal from the Kappa camera did not correspond one to one with the three fluorochrome signals, a careful calibration was necessary. The method described herein can be applied to all dye combinations. The results are shown for DAPI, FITC, and Texas red, because they form a typical CGH dye combination. In principle, this calibration has to be done for all dye combinations, filters, and gain setups; this might be seen as a disadvantage in working with the color camera. A cytogenetic laboratory would normally use a standard hybridization technique in routine application, however, with one dye combination for all cases in order to maintain the comparability of results. This would considerably reduce the calibration workload. Moreover, cross-talks between different filters may appear when using a B&W camera, so calibration may be necessary in this case too.

Another argument that is often used against color cameras is that they eliminate the ability to correct for magnification changes. Optical magnification differences between the different colors cause a shift of several pixels between the center and the edge of an image. This can be easily corrected for B&W cameras.

With the Kappa CF 15 MC system, this pixel shift was on the order of two pixels between blue (DAPI) and red (Texas red) for 250 pixels between center and edge of an image. This effect was linear with the wavelength (data not shown). For example, assuming an exactly linear chromosome located in the center of the field of view with a length of less than 10 µm, which corresponds to fewer than 100 pixels for the objective magnification used, this effect would be about one pixel. This is comparable to the segmentation error for a chromosome. However, this effect becomes worse towards the edges of the image. Because the ratio of a green color (e.g., FITC) to a red color (e.g., Texas red) is usually determined during CGH analysis, the effect is always less than or approximately one pixel for these two colors under the magnification conditions used, even in locations extremely near the edge. After averaging a number of chromosomes for CGH, this one pixel effect becomes negligible.

The dynamic range of the Kappa CF 15 MC camera is limited to 8 bits. Nowadays, many B&W cameras have dynamic ranges of 10, 12, or 14 bits (e.g., the Photometric camera used 12 bits). These cameras require compatible frame grabbers, which work with the same dynamic range. In working with off-the-shelf frame grabbers, the TIFF format has in the last few years become a standard method for writing images into files for subsequent analysis. In principle, TIFF supports any pixel precision. This was also found for the hardware and software configuration used in combination with the Photometries B&W camera. However, du Manoir et al. (9) recently defined requirements for

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<tr>
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hardware and software capabilities of systems used for CGH analysis. When fulfilled, these will permit a level of performance in the analysis that is comparable to the best current practice. The authors found that for CCD cameras a "...resolution of 8 bits is sufficient..." Moreover, well established programs (10,18) used in combination with 12-bit cameras perform a conversion from a 12-bit to an 8-bit range before image processing. In considering the use of such systems, the advantage of 12-bit image acquisition appears doubtful. In our experience, an 8-bit dynamic range is quite sufficient for most applications in FISH.

The linearity of the camera should also be considered in this context B&W cameras are known to have a high linearity. This was also true for the Kappa camera if the linearity was measured separately for R, G, and B. In that case, usually only one color channel worked linearly within the whole range, whereas the two others reached a level of saturation. Insofar as the gain settings for the three colors are not independent of each other, simultaneous linearity for all three colors can be obtained for only one color within the entire dynamic range. The other two colors do not reach their maximum intensity with the same gain settings. For CGH imaging using a green dye (e.g., FITC) and a red dye (e.g., Texas red), this effect was relatively small. G and R were simultaneously linear within a sufficient range (for comparison, see Fig. 3).

In conclusion, the Kappa CF 15 MC color CCD camera can be used for CGH imaging. The software implemented in Optimas makes the system easy to handle on a PC supporting Microsoft Windows. The color system is not faster in practice owing to its lower sensitivity, despite the fact that B&W cameras require sequential image acquisition. The image quality of expensive B&W cameras is superior to that of most color cameras developed so far. The suitability of the Kappa CF 15 MC for analyzing extremely small and low-light-level details of FISH signals (e.g., single copy genes) other than CGH would be limited. It offers an alternative and cost-effective approach, however, for routine CGH using a standard highly reproducible hybridization protocol with a standard set off fluorochromes.

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LITERATURE CITED: