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ARTICLES

New strategy for multi-colour fluorescence *in situ* hybridisation: COBRA: COmbined Binary RAtio labelling

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Multicolour *in situ* hybridisation (MFISH) is increasingly applied to karyotyping and detection of chromosomal abnormalities. So far 27 colour analyses have been described using fluorescently labelled chromosome painting probes in a so-called combinatorial approach. In this paper a new strategy is presented to use efficiently the currently available number of spectrally separated fluorophores in order to increase the multiplicity of MFISH. We introduce the principle of COBRA (COmbined Binary RAtio labelling), which is based on the simultaneous use of combinatorial labelling and ratio labelling. Human chromosome painting in 24 colours is accomplished using four fluorophores only. Three fluorophores are used pair wise for ratio labelling of a set of 12 chromosome painting probes. The second set of 12 probes is labelled identically but is also given a binary label (fourth fluorophore). The COBRA method is demonstrated on normal human chromosomes and on a lymphoma (JVM) cell line, using probes enzymatically labelled with fluorescein, lissamine and cy5 as primary fluorophores, and diethylaminocoumarin (DEAC), a blue dye, as combinatorial fourth label to demonstrate incorporated digoxigenin. In addition, the principle was tested using chemical labelling. The first set of 12 painting probes was therefore labelled by ULS (Universal Linkage System), using DEAC, cy3 and cy5 as primary labels, and the second set was labelled similarly, but also contained a digoxigenin-ULS label, which was indirectly stained with fluorescein. Subsequently, a mathematical analysis is presented and methods are indicated for achieving an MFISH multiplicity of 48, 96 or even higher using existing technology.

Keywords: cytogenetics; chromosomal aberrations; multicolour FISH; combinatorial labelling; chemical labelling; ratio labelling; digital fluorescence microscopy

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Introduction

The introduction of fluorescence *in situ* hybridisation (FISH) has significantly changed cytogenetics. PCR amplification of DNA from human chromosomes obtained by flow sorting or by micro-manipulation allows generation of probe sets that specifically paint one type of chromosome or parts thereof. Recently, multicolour FISH using 24 chromosome specific painting probes was used to produce a full FISH karyogram.[1,2]

FISH karyotyping is now successfully applied to elucidate complex chromosome rearrangements.[3] Multicolour FISH analysis of chromosomes is not necessarily restricted to the use of whole chromosome paints. Recently, sets of probes have been generated that specifically recognise the (sub)telomeric regions of a particular chromosome and are applied in a multicolour FISH format to detect cryptic translocations, frequently occurring in mental retardation.[4] Moreover, multicolour FISH using sub-regional DNA probes created by microdissection or isolated from human/rodent somatic cell hybrids allows for artificially banded (bar coded) chromosomes.[5,6] The number of bands in such FISH stained chromosomes depends on how many probes one may want to generate; there are, however, in principle, no limitations to recognising as many bands as are seen in Giemsa banded chromosomes (about 800).

The selective staining of 24 human chromosomes is at present accomplished by binary combinations of probes that are labelled with five distinct fluorophores.[1,2,7] For this so-called combinatorial labelling (also called multiplex FISH[2]) the number of recognisable targets (n) using (k) different fluorophores is $n = 2^k - 1$ colours.[8] Five fluorophores thus allow a maximum of 31 colours, sufficient to recognise 24 chromosomes, but insufficient for instance to explore the use of p and q arm specific probes for the detection of intrachromosomal rearrangements. Thus multicolour FISH analysis of chromosomes would benefit directly from a method of increasing the number of simultaneously recognisable targets beyond the 27 reported so far.

Higher FISH multiplicity using the binary approach is feasible by increasing the number of fluorophores to six. Since at present the available spectrum for five fluorophores is already utilised from the UV to the near infra-red, introduction of a sixth dye may lead to increased spectral overlap and undesirable cross talk between detection channels. More narrow excitation and emission filters may circumvent that problem, but inevitably lead to longer exposure times because of the reduced number of available photons. Alternatively, higher FISH multiplicity is achievable by ratio labelling. This technique, by which a given probe is composed of a mixture of probes with different fluorescent labels, has great potential. As an illustration, one may consider the number of recognisable colours that could be composed from the three primary colours blue, green and red. In practice, however, ratio labelling is considerably more complex than combinatorial labelling. Recognition of chromosomes stained with ratio labelled probes is not a 'yes or no' colour decision (as in the binary approach) but requires accurate measurement of colour.

The present paper explores the advantages of both methods, emphasising practical considerations and limitations. A strategy is described to achieve a FISH multiplicity of 48, 96 or more. It is based on the strategic combination of binary labelling and ratio labelling, utilises existing technology and requires no major investment in microscope hardware other than a good digital fluorescence microscope.

Materials and Methods

Principle of COBRA: COmbined Binary RAtio labelling

The principle of COBRA is based on the simultaneous use of combinatorial (ie binary) labelling and so-called ratio labelling.[9–11] A number of spectrally separated fluorophores is used for ratio labelling, in such a way that maximally two fluorophores are used to produce a certain colour. When this is applied for three fluorophores, and each pair of fluorophores results in five colours, a total of 12 colours is achieved (lower triangle in Figure 1). This primary probe set is directly fluorescently labelled using methods such as nick translation and labelling by DOP-PCR or chemical labelling.[12,13] A second set of 12 probes, recognising different targets is labelled in exactly the same way, but in addition is given a hapten (for instance biotin or digoxigenin). This hapten is developed using avidin or antibodies labelled with a fourth fluorescent label, spectrally easily distinguishable from the three primary fluorophores used for ratio labelling. Thus, the set of 12 is multiplied by two, which results in 24 colours using four fluorophores only (two middle triangles in Figure 1), which is one fluorophore less than reported so far to accomplish staining of the 24 human chromosomes. Extra 'free' fluorophores may be used to repeat this process, exploring a second hapten, which again results in a doubling of the number of achievable colours (giving 48 colours) (upper triangles in Figure 1).

Obviously, even greater increments in the number of colours are achievable if more than 12 primary colours are produced in the basic triangle, either by using more than three fluorophores or by distinguishing more ratios.

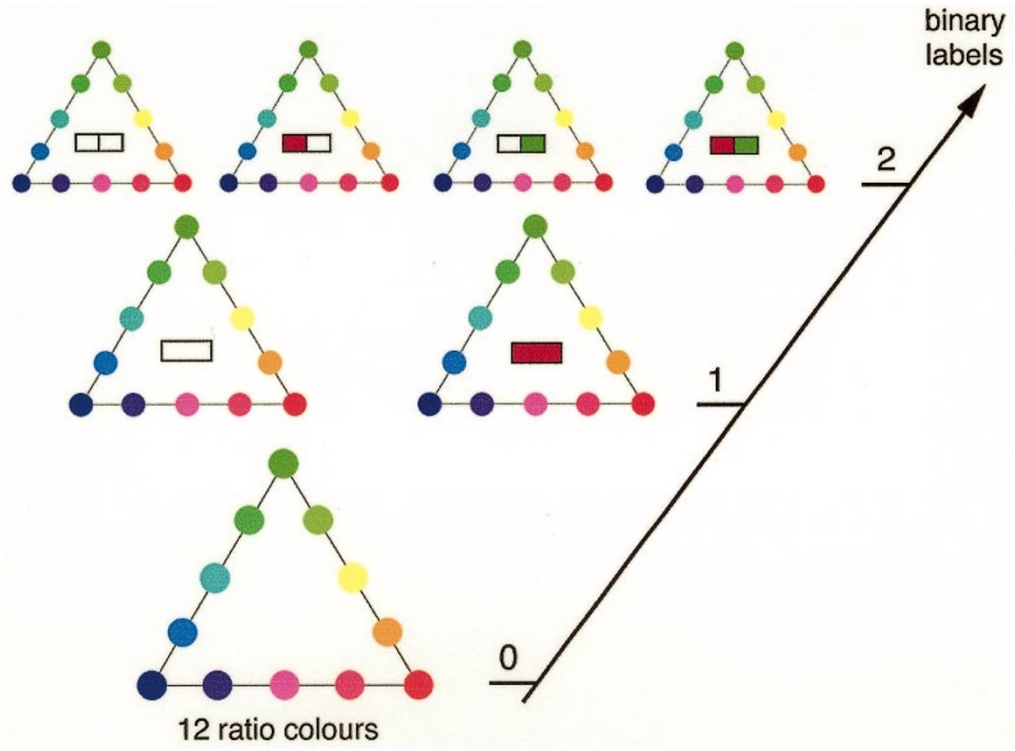

Figure 1 *Principle of COBRA. The primary set of 12 ratio colours is doubled each time an independent binary label is introduced, resulting in 24 colours for 1 hapten, and in 48 colours for 2 haptens*

Mathematically, the total number of achievable COBRA colours is described as follows. Assume that *n* fluorochromes are used for ratio labelling and only two of those fluorochromes are simultaneously used per target, while additionally *m* fluorochromes can be binary labelled to the same target and *r* ratios can be resolved for ratio labelling, then the number of different colours that can be distinguished is given by the following formula:

$$\text{No. of colours} = \left(n + \frac{r \times n!}{2!(n-2)!}\right) \cdot 2^m$$

where

n

r

m

Examples:

a. 2 fluorochromes for ratio labelling ($n = 2$), 0 ratios ($r = 0$) and 0 binary label ($m = 0$) results in 2 colours, as expected;

b. 3 fluorochromes for ratio labelling ($n = 3$), 3 ratios ($r = 3$) and 1 binary label ($m = 1$) results in 24 colours (the situation that will be demonstrated in this paper);

c. Increasing the number of ratios to $r = 4$ and the number of fluorochromes for ratio labelling to 4, results in 28 colours;

d. Each binary fluorochrome results in doubling of the number of colours; that is to 56 (for 1) or to 112 (for 2).

The principle of this concept is demonstrated on 24 human chromosomes using enzymatic labelling of probes and probe mixing to accomplish ratio labelling, as well as direct attachment of the colour code to the probes using chemical labelling. For the enzymatic labelling we used fluorescein, lissamine and cy5 as the three basic colours, and diethylaminocoumarin (DEAC), a blue fluorophore, as fourth fluorophore. For chemical labelling DEAC, cy3 and cy5 served as primary fluorophores, and fluorescein was used as the fourth binary label.

Multicolour FISH Staining of Human Chromosomes

Preparation of human metaphase chromosomes was performed as described by Wiegant *et al*.[12,13] Chromosomes from normal human individuals as well as from *in vitro* cultured JVM-2 cells were used.[14] Dr Nigel Carter (Sanger Institute, Cambridge, UK) kindly provided the probes for chromosomes 1–8, 13–20, 22 and X. In addition, probes for all chromosomes were obtained from Cytocell, Adderbury, Banbury, UK. All probe DNA was amplified by DOP-PCR[15] to generate a set of painting probes for all 24 human chromosomes. For the enzymatical labelling, approach probes for 1–8, 13–20, 22 and X from Dr Nigel Carter were supplemented with probes for chromosomes 9–12, 21 and Y from Cytocell. For the chemical labelling approach only probes from Cytocell were used.

Table 1

Chrom. No.	*Fluorescein (%)*	*Lissamine (%)*	*Cy 5 (%)*	*Dig (%)*
1	300	0	0	0
7	150	50	0	0
Y	60	50	0	0
19	85	150	0	0
3	0	300	0	0
9	0	75	40	0
15	0	100	150	0
21	0	70	150	0
5	0	0	300	0
11	35	0	80	0
17	120	0	150	0
13	150	0	75	0
4	300	0	0	100
10	160	40	0	100
16	110	100	0	100
22	80	150	0	100
6	0	300	0	100
12	0	80	35	100
18	0	100	100	100
X	0	90	150	100
2	0	0	300	100
20	80	0	150	100
14	120	0	120	100
8	150	0	50	100

Note: 100% is equal to 100 ng of labelled probe

Enzymatic Labelling of Probes All probes were fluorescently labelled by incorporation of labelled dUTPs either by PCR or nick translation using fluorescein-, digoxigenin-dUTP (all from Boehringer Mannheim, Germany), lissamine-dUTP (NEN Life Science Products, Boston, MA, USA) or cy5-dUTP (Amersham, Amersham, UK). The digoxigenin-labelled probes were detected indirectly using diethylaminocoumarin (DEAC) (Molecular Probes, Eugene, OR, USA). For labels and identification of the various probes see Table 1.

Chemical Labelling of Probes using ULS (Universal Linkage System) Chemical labelling of probes was performed using the Universal Linkage System (ULS) (Kreatech Diagnostics, Amsterdam, The Netherlands). DEAC, cy3 and cy5 were chosen as primary fluorophores and fluorescein as combinatorial fourth label to demonstrate digoxigenin-ULS (dig-ULS) labelled probes.[16] The following strategy was used to label and dissolve the ULS-labelled probe set.

First, chromosome-specific painting probes for chromosomes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and X (100–400 ng) were labelled in one reaction with dig-ULS according to the manufacturers' instructions. Thereafter, this probe set was purified on a Qiagen quick spin column (Qiagen Inc, Valencia, CA, USA) according to the manufacturers' instructions. The labelled probe mixture was eluted from the Qiagen column using 100 µl of 10 mM Tris HCl pH 8.5.

Second, all chromosome-specific painting probes were fluorescently labelled according to Table 2 by mixing 30 µl of the listed ULS compounds (or mixtures thereof) with 1 µg of chromosome-specific painting probe DNA (all from Cytocell) using DEAC-ULS (26.7 µM), cy3-ULS (20 µM) and cy5-ULS (13.3 µM) in a final volume of 100 µl of water. In case probes were labelled with mixtures of two different ULS compounds, the ULS compounds were first mixed in the desired ratio before the probe DNA was added. After 15 min incubation at 65°C, the labelled probes were purified on Qiagen quick spin columns. The labelled probes were eluted from the Qiagen columns using 100 µl of 10 mM Tris HCl pH 8.5. Prior to the hybridisation, fluorescent ULS-labelled probes were combined in amounts as indicated in the right-hand column of Table 1 together with the 100 µl of dig-ULS labelled probe mixture from the first step. This probe mixture was then ethanol-precipitated in the presence of 10 × excess low molecular weight fish sperm DNA (Boehringer Mannheim), and 3 × excess human C_0tl-DNA (Gibco, BRL, Paisley, UK). Thereafter the probe mixture was dissolved in 10 µl 50% deionised formamide, 2 × SSC, 50 mM sodium phosphate pH 7, 10% dextran sulphate. This 10 µl of probe mixture was used as hybridisation solution (see later).

FISH Staining of Human Metaphase Chromosomes Hybridisation conditions of enzymatically and ULS labelled probes were similar. Slides with metaphase chromosomes were pretreated with RNaseA and pepsin according to Wiegant *et al*.[17] The chromosome preparations were denatured by incubating them for 90 sec at 80°C in 60% formamide, 2 × SSC, pH 7 on a hotplate. After removal of the coverslip the slides were dehydrated through an ethanol series and air dried. Then, 10 µl hybridisation mixture was applied under a 18 × 18 mm coverslip, sealed with rubber cement, and hybridisation was performed for 120 hr at 37°C in a humid chamber. In case enzymatically labelled probes were used, the hybridisation mixture contained 50% formamide, 2 × SSC, 50 mM sodium phosphate pH 7, 10% dextran

Table 2

Chrom. No.	*µl DEAC-ULS (26.7 µM)*	*µl Cy3-ULS (20 µM)*	*µl Cy5-ULS (13.3 µM)*	*ng probe DNA in hybrid. mix*
1	30	0	0	500
2	0	0	30	300
3	0	30	0	400
4	30	0	0	500
5	0	0	30	600
6	0	30	0	500
7	22.5	9	0	300
8	22.5	0	9	400
9	0	22.5	7.5	500
10	22.5	9	0	500
11	7.5	0	22.5	500
12	0	22.5	7.5	400
13	22.5	0	9	300
14	19.5	0	15	300
15	0	18	15	300
16	15	18	0	300
17	19.5	0	15	300
18	0	18	15	400
19	7.5	22.5	0	400
20	7.5	0	22.5	400
21	0	10.5	22.5	300
22	7.5	22.5	0	400
X	0	10.5	22.5	400
Y	15	18	0	100

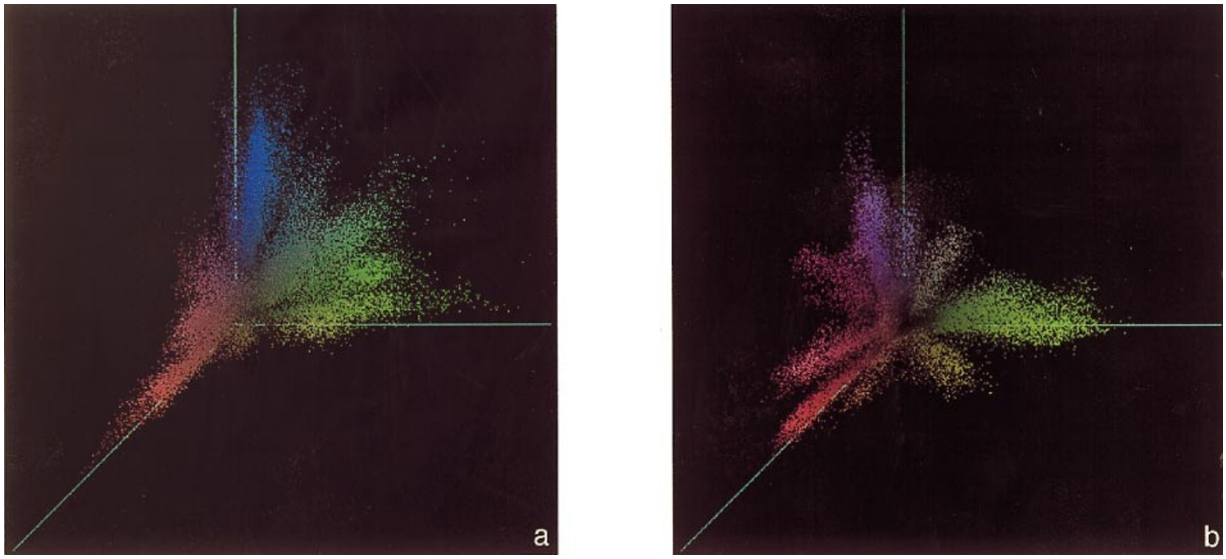

Figure 2 *Human chromosomes were stained in 24 colours using the COBRA principle. For each of the 24 chromosomes the fluorescence intensity was plotted in a three-dimensional colour space. Each coloured dot represents the measured colour intensity of an image point (pixel) of a certain chromosome. (***a***): three primary colours (fluorescein, lissamine, cy5); without binary DEAC label; (***b***): idem, with binary DEAC label.*
Note: Figure 1 is a schematic top view of the 2 × 12 clusters seen at equal x, y, z values of the measured data shown in this figure

sulphate, 300 ng of each fluorescein-, lissamine- and cy5-labeled probe (single labelled probes), 100–250 ng of each ratio labelled probe, 100 ng of each digoxigenin labelled probe, 3 × excess human C_0tl-DNA and 10 × excess low molecular weight fish sperm DNA in 10 μl. When chemically modified probes were used, the hybridisation mixture contained 50% formamide, 2 × SSC, 50 mM sodium phosphate pH 7, 10% dextran sulphate, 100–500 ng of each DEAC-, cy3- and cy5-labelled probe (both single and ratio labelled probes) (see Table 2), 100–400 ng of each dig-ULS labelled probe, 3 × excess human C_0tl-DNA and 10 × excess low molecular weight fish sperm DNA in 10 μl. Before application, the probes were denatured for 10 min at 80°C, followed by 60 min incubation at 37°C to allow pre-annealing with the 3 × excess of C_0tl-DNA.

After a 10 min post-hybridisation wash in 2 × SSC/0.1% Tween 20 at 37°C to remove the coverslips, the slides were washed 2 × 5 min in 50% formamide, 2 × SSC, pH 7 at 44°C. This was followed by 2 washes (5 min each) in 0.1 × SSC at 60°C and a 5 min wash at RT in TNT (0.1 M Tris HCl pH 7.4, 0.15 M NaCl, 0.05% Tween 20). The enzymatically labelled dig probes were detected with a mouse monoclonal antibody against digoxin (Sigma) followed by a sheep anti-mouse antibody conjugated to DEAC. The chemically labelled dig probes were detected with a mouse monoclonal antibody against digoxin (Sigma) followed by a rabbit anti-mouse antibody conjugated to FITC (Sigma, St Louis, USA). Chromosomes were counterstained with DAPI. The slides were embedded in Vectashield (when enzymatically labelled probes were used) or Citifluor (Agar, Stansted, UK) (when chemically labelled probes were used) prior to microscopical evaluation.

Digital Imaging Microscopy

Digital fluorescence imaging was performed using a Leica DM-RXA epifluorescence microscope (Leica, Wetzlar, Germany) equipped with a 100-W mercury arc lamp and computer controlled filter wheels with excitation and emission filters for visualisation of DEAC, fluorescein, lissamine, cy3 and cy5, using HQ-FITC, Pinkel set plus SP 570, HQ-Cy3, HQ-Cy5 and DEAC filter (Chroma Technology, Brattleboro, VT, USA) respectively. DAPI was excited with UV light using block A. A 63 × objective (N.A. 1.32, PL APO, Leica) was used. Image acquisition and analysis was performed on a Cytovision workstation (Applied Imaging, Newcastle, UK). This system consists of a PC (Pentium 133 MHz processor, 24 Mb Ram, 2.1 Gb disc and 17 in display) interfaced to a Coolview camera (Photonic Science, Robertsbridge, UK). The camera has thermo-electric cooling, which allows on chip integration up to circa 30 sec. Images are digitised in an 8-bit 768 × 512 image format.

Image acquisition was performed as described before.[11] Chromosomes were segmented interactively by thresholding the DAPI image. The segmented image was used as a mask for the colour image, which was composed of the three images corresponding with the ratio labelled fluorochromes (green for fluorescein, red for lissamine and blue for cy5) and of the DEAC image. Note that this procedure does not require thresholding of the three colours. The fourth DEAC image was evaluated binary, that is chromosomes with or without DEAC fluorescence were distinguished. This was performed by finding the optimal threshold in the histogram of the DEAC image for the pixels lying within the DAPI mask. Typically, two gaussion distributions were observed, corresponding to DEAC positive and negative chromosomes.

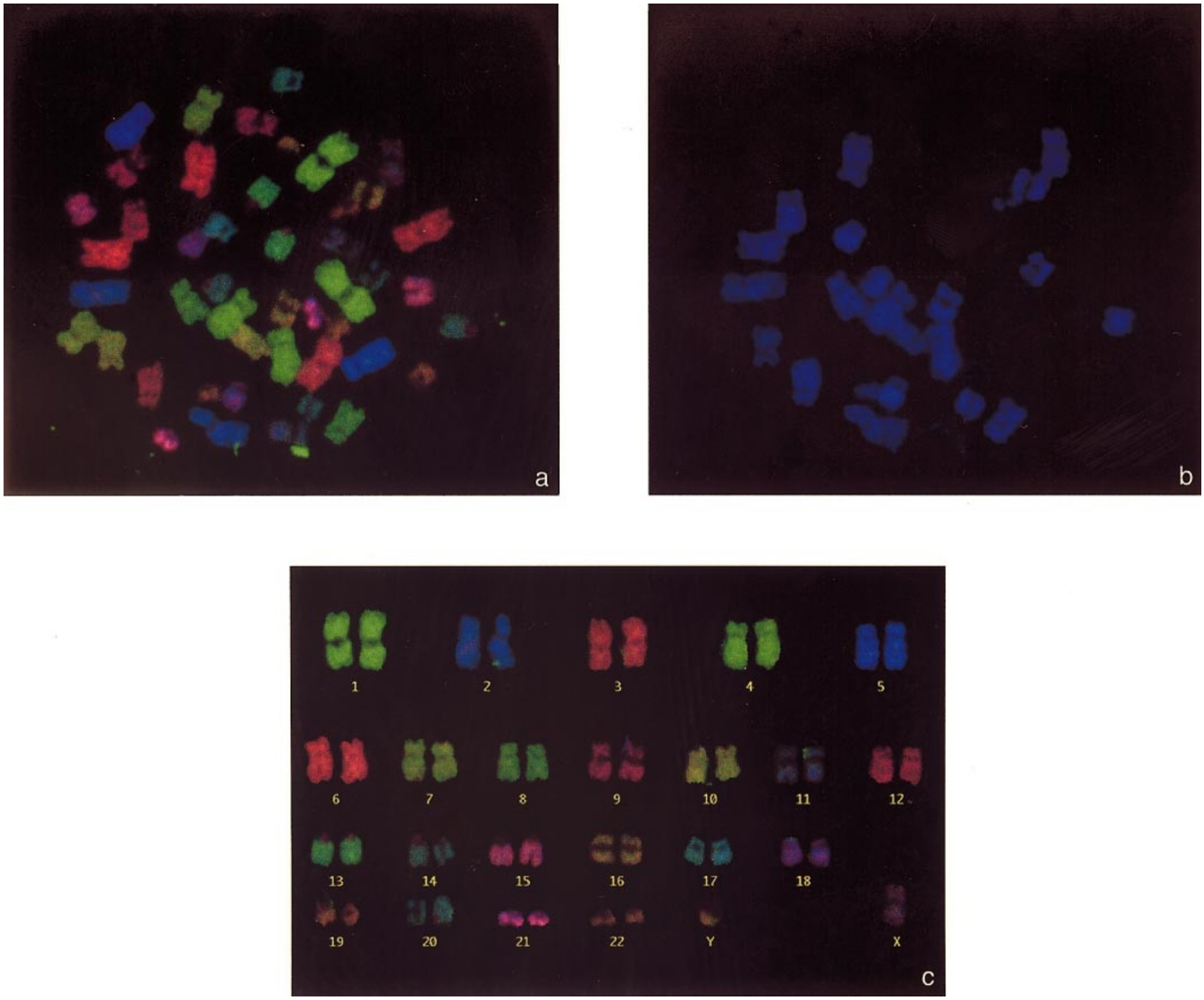

Figure 3 *Normal human chromosomes labelled by COBRA in 24 colours (same data as Figure 2). (***a***) Image (12 colours) resulting from the primary dyes used in ratio labelling; (***b***) DEAC image of the same metaphase cell; (***c***) Karyogram resulting from the combination of image (***a***) and (***b***) and automated classification*

Samples stained with ULS labelled probes were analysed similarly, in the sense that the ratio images related to DEAC (green), cy3 (red) and cy5 (blue), and the binary fourth image to fluorescein.

Classification was performed in two steps: the chromosome classification was followed by a pixel classification to detect possible translocations. Chromosome classification was based mainly on the modal colour value of each chromosome, eg its position in one of the colour triangles (the one with or without the binary label), as shown in Figure 1. The shortest distance of the measured modal colour value of a chromosome to the theoretical expected ratio colour of all chromosome classes was therefore calculated. In order to compensate for non-specific fluorescence contributions and to increase the robustness of the method the theoretical expected colour values were warped on to a triangle formed by the measured modal values of the chromosomes with only one ratio colour. Besides the modal colour value the length of the chromosomes was also used for classification. Theoretically, the colour values of the chromosomes should correspond to the original probe ratios. In practice, however, a more robust approach is obtained, when a number of metaphases is used for training the classifier (Vrolijk *et al*, manuscript in preparation). Following object classification, each pixel within a chromosome was classified on the basis of the shortest distance to the measured chromosome classes. The binary (fourth colour) information of each pixel was used to decide within which colour triangle distance calculations should be

performed. Assignment of classification colours is considered useful and foreseen, but was not implemented in the current software.

Finally, a karyogram was generated based on chromosome classification showing the ratio colours, as described above. A karyogram, in which a pseudo colour was assigned to the corresponding chromosome class of each separate chromosome pixel was produced to facilitate the interactive detection of chromosome translocations. When needed the DAPI banding image was used for comparison purposes.

Results

A 24 colour COBRA staining procedure using four fluorophores was applied to normal and abnormal chromosomes. The optimal conditions for labelling the probes and the final composition of the probe set required some fine tuning due to the fact that some probes performed better than others. Typically, lower performing FISH probes were given colour combinations such that colour overlap with other probes was minimised.

Optimal staining results were obtained at prolonged hybridisation times (5 days), although 3 days in many cases was sufficient. The use of C_0tl-DNA was found essential for selective staining of chromosomes; the optimal amount per incubation mixture was a $3 \times$ excess.

Figure 2 shows how the 24 chromosomes occupy the colour space. Typically, within a certain chromosome image, signal intensities showed relatively large variations, due to local differences in FISH intensity. The characteristic colour, however, was sufficiently constant to form clusters, with a defined angle within the 3-D colour space (Figure 2). Although some chromosome clusters showed overlap, they were well enough separated to be classified automatically using the procedure described above.

Figure 3 shows the actual chromosome images and the resulting karyogram. Integration times varied depending on the fluorophore used and ranged from 0.5 to 20 sec. An entire COBRA acquisition and analysis procedure typically took approximately 1 min. Applied to abnormal chromosomes as shown in the JVM cell line, COBRA allowed for easy detection of abnormal chromosomes (Figure 4). The results shown in Figures 2–4 were obtained with enzymatically labelled probes, whereby ratio labelling was accomplished by mixing probes in desired proportions. The same COBRA principle was then applied to probes that were labelled chemically using ULS. In this method it is essential that in principle each probe molecule contains the ratio code, making mixing obsolete. Although ULS modifications of fluorescein, rhodamine and cy5 performed adequately for single colour chromosome staining, stable ratios were not achieved. This is probably due to strongly different reaction kinetics of the ULS derivatives, and/or to physical interactions of the DNA bound dye molecules. Ratio labelling of DEAC, cy3 and cy5 performed much better, and could

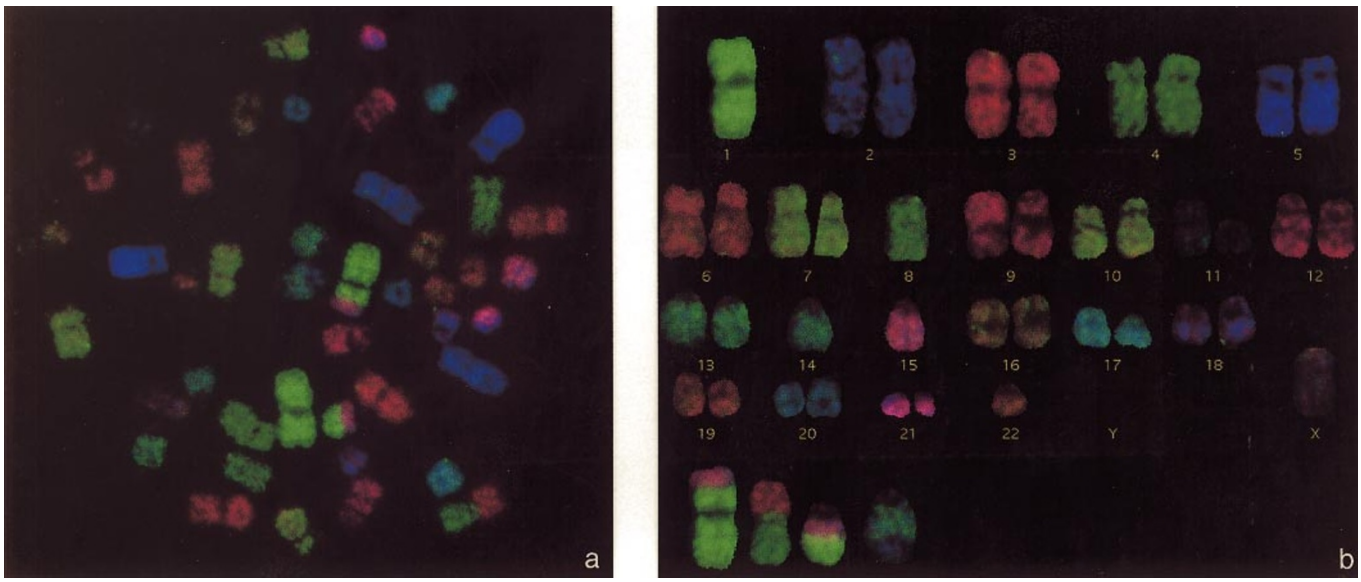

Figure 4 *COBRA (24 colours) applied to a JVM cell line (B-prolymphocytic leukemia) showing translocations t(11,14),t(3,8) and t(1,15)*

be well combined with binary fluorescein labelling. Results obtained with these probes are shown in Figure 5.

The robustness of COBRA depended on the quality of the metaphase chromosomes obtained, as is the case for automated analysis of both Giemsa-banded and

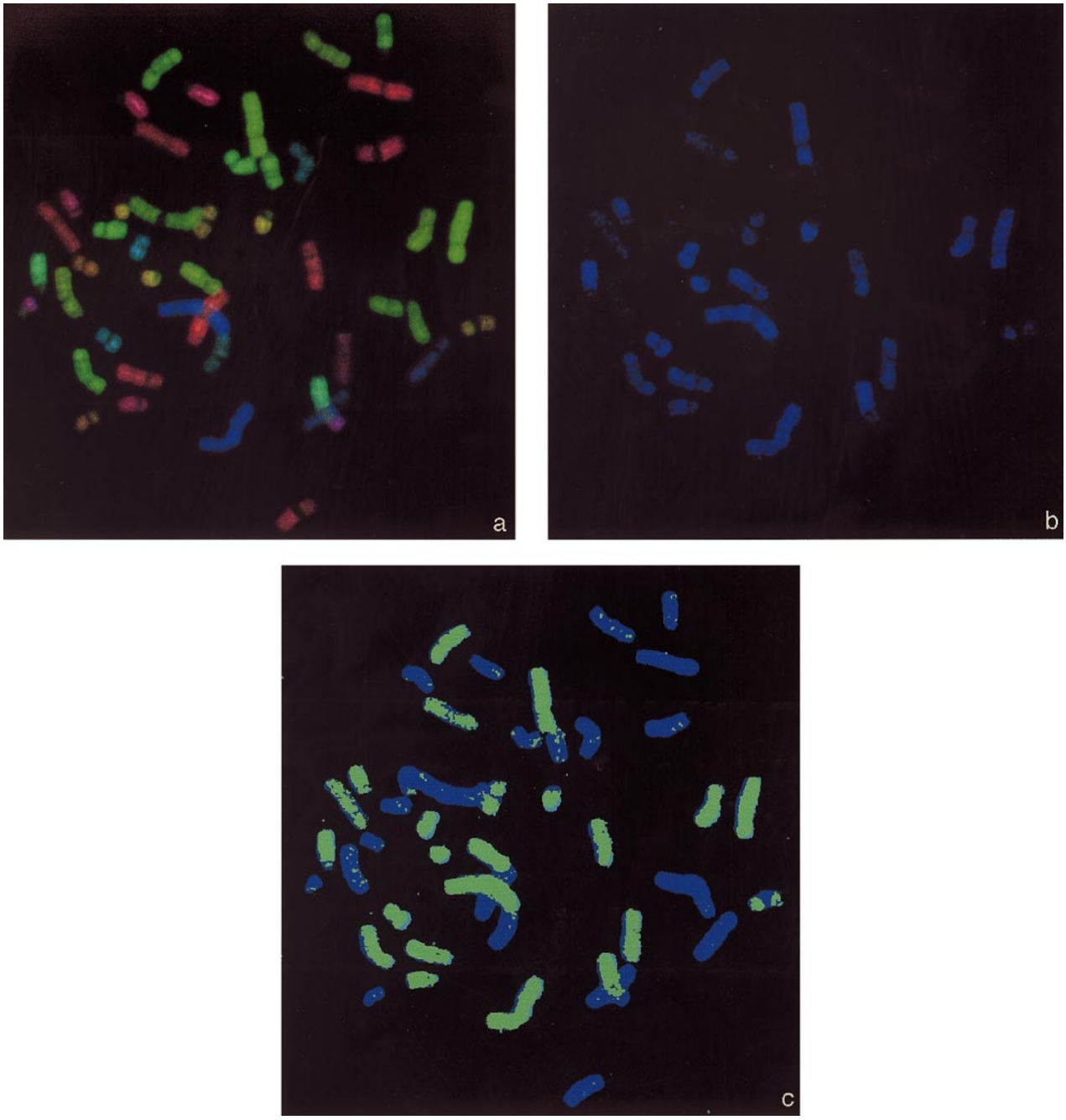

Figure 5 *Results of ratio labelling obtained using chemical labelling (ULS system). The fluorophores DEAC, cy3 and cy5 were used as primary labels for ratio labelling. The dig-ULS labelled second set of 12 probes was demonstrated indirectly using fluorescein labelled immunoconjugates.* (**a**) *Image (12 colours) resulting from the three primary dyes used for ratio labelling;* (**b**) *Image of the binary (fourth) label (fluorescein, but shown in blue false colour);* (**c**) *Thresholded image* (**b**) *to identify the fluorescein positive and negative sets of chromosomes: Note: DEAC is used as direct probe label here (by ULS), whereas in Figures 2, and 4 it was used as binary label (as immunoconjugate)*

FISH-stained chromosomes. Good quality slides always resulted in images of good signal-to-noise ratio that could be classified automatically, whereas user intervention increased with decreasing staining quality.

Discussion

Despite its only recent introduction, multicolour FISH karyotyping has changed cytogenetics significantly. Most studies reported so far use five different fluorophores in a combinatorial approach, which allows for a theoretical maximum of 31 colours.

As has been shown, colours may also be created by mixing the same kind of probes, but now in different proportions to create various ratios of two or more kinds of fluorophores.[6,12] These studies have shown that six or seven ratios of two different fluorophores can be distinguished. Ratio labelling therefore has great potential to increase the number of colours. However, its applications so far are limited, due to the fact that recognition of combinatorially labelled chromosomes is based on thresholding of the signal only (binary decision), whereas ratio labelled chromosomes require accurate measurements. If colours are created by mixing differently labelled probes, the variation in signal intensity and/or colour may be large due to variations in hybridisation efficacy as a function of the type of label. This could be overcome by avoiding mixing differently labelled probes and providing each DNA molecule with the various labels, achievable using enzymatic or chemical labelling procedure. A problem that may occur is the physical interaction of the different fluorophores bound to the same DNA molecule, causing energy transfer or quenching, which may result in poorly reproducible labelling. These potential problems cause MFISH so far to be based on combinatorial labelling using mixtures of probes.

The COBRA principle described in this paper combines the advantages of both approaches. It 'settles for' making ratios of two fluorophores only, but utilises the possibility of doubling the number of colours by introducing indirectly labelled haptens, which require a binary decision only. As shown here, this approach is feasible and allows for identifying 24 human chromosomes using four fluorophores only. Moreover, the use of Cy7 an infra-red fluorescing dye is avoided, which allows for the use of less expensive cameras. The full potential of this approach has not yet been explored. So far only painting probes have been used in COBRA. Considering the short exposure times, we anticipate that other types of probe such as YACs or PACs can be used in a similar approach.

As the mathematical equation shows, the number of colours particularly increases if more dyes or more ratios are used for the primary colour set. It has been shown that distinction of a ratio of 6 or 7 of two dyes is feasible.[10]

We anticipate that such an approach is best achieved if chemical labelling is used. Chemical labelling methods, such as ULS, may be advantageous for large scale production of quality controlled painting probes. Further exploration of multicolour FISH analysis in cytogenetics seems at present to depend more on the availability of quality controlled probes than on further development of FISH microscope workstations. Although some refinement is needed, currently available hardware and software is well suited for MFISH. In this context the COBRA strategy for efficient use of fluorophores can significantly contribute to a further increase of MFISH multiplicity and thereby to further exploitation in cytogenetics.

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