Technical Advance

Multicolor-FICTION

Expanding the Possibilities of Combined Morphologic, Immunophenotypic, and Genetic Single Cell Analyses

José Ignacio Martín-Subero,^{*†} Ilse Chudoba,[‡]
Lana Harder,^{*} Stefan Gesk,^{*} Werner Grote,^{*}
Francisco Javier Novo,[†] María José Calasanz,[†]
and Reiner Siebert^{*}

From the Institute of Human Genetics,^{} University Hospital Kiel, Kiel, Germany; MetaSystems,[‡] Altlußheim, Germany; and the Department of Genetics,[†] University of Navarra, Pamplona, Spain*

Phenotypic and genotypic analyses of cells are increasingly essential for understanding pathogenetic mechanisms as well as for diagnosing and classifying malignancies and other diseases. We report a novel multicolor approach based on the FICTION (fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms) technique, which enables the simultaneous detection of morphological, immunophenotypic, and genetic characteristics of single cells. As prerequisite, multicolor interphase fluorescence *in situ* hybridization assays for B-cell non-Hodgkin's lymphoma and anaplastic large-cell lymphoma have been developed. These assays allow the simultaneous detection of the most frequent primary chromosomal aberrations in these neoplasms, such as t(8;14), t(11;14), t(14;18), and t(3;14), and the various rearrangements of the *ALK* gene, respectively. To establish the multicolor FICTION technique, these assays were combined with the immunophenotypic detection of lineage- or tumor-specific antigens, namely CD20 and ALK, respectively. For evaluation of multicolor FICTION experiments, image acquisition was performed by automatic sequential capturing of multiple focal planes. Thus, three-dimensional information was obtained. The multicolor FICTION assays were applied to well-characterized lymphoma samples, proving the performance, validity, and diagnostic power of the technique. Future multicolor FICTION applications include the detection of preneoplastic lesions, early stage and minimal residual diseases, or micro-metastases. (Am J Pathol 2002, 161:413–420)

Phenotypic and genetic studies of human neoplasias have widely contributed to our current understanding of carcinogenesis and to the clinical management of affected patients.¹ The integration of different disciplines allowing a comprehensive characterization of cancer cells is becoming increasingly important in routine diagnostics and research of malignant tumors.^{2,3} The genetic analysis of single cells simultaneously to their morphological and immunophenotypic characterization is heavily hampered by the fact that conventional cytogenetic-, as well as polymerase chain reaction-, blotting- or CHIP-based techniques usually require the destruction of the cell. In contrast, fluorescence *in situ* hybridization (FISH) on interphase nuclei preserves cell structure. Moreover, FISH-based interphase cytogenetics has been repeatedly shown to be more reliable than polymerase chain reaction or conventional cytogenetic methods to detect cancer-associated chromosomal aberrations.^{4–7}

The FISH technique applies fluorescent probes that hybridize to their cellular counterparts leading to fluorescent signals at the place where the cellular target sequence is located. Depending on the probe design, all types of chromosomal aberrations, eg, deletions, amplifications, or translocations, can be rapidly detected and quantified.⁸

In 1992, Weber-Matthiesen and co-workers⁹ developed a technique combining FISH with fluorescence immunophenotyping referred to as FICTION (fluorescence immunophenotyping and interphase cytogenetics as a tool for the investigation of neoplasms). This method allows the simultaneous study of morphological, immunophenotypic, and genetic features of single cells. So far, the applications of FICTION have been hindered by the

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Address reprint requests to José Ignacio Martín-Subero, Ph.D., Institute of Human Genetics, University Hospital Kiel, Schwanenweg 24, 24105 Kiel, Germany. E-mail: imartin@medgen.uni-kiel.de.

restriction to three fluorescent dyes and the frequent use of indirect FISH probes. Thus, no more than two chromosomal loci could be studied simultaneously. The recent availability of many new fluorescent dyes, along with improvements in digital imaging has led to the development of multicolor FISH techniques for karyotyping such as spectral karyotyping (SKY)¹⁰ and multifluor FISH (M-FISH).¹¹ These methodologies now provide the prerequisite to overcome the limitations of conventional FISH.

We present an improved FISH technique, termed multicolor FISH (M-FISH), which in addition to assessment of morphology and fluorescence immunophenotyping allows the simultaneous investigation of multiple chromosomal aberrations. Two different assays were established as proof of the M-FISH principle, one for the study of B-cell non-Hodgkin's lymphomas (B-NHLs)—the most prevalent subtype of malignant lymphomas—and another for anaplastic large-cell lymphomas (ALCLs).

B-NHLs comprise a heterogeneous group of malignancies.¹² These tumors regularly express B-cell markers such as CD20 and CD79a rendering these antigens targets for immunophenotypic detection. Following the new World Health Organization classification for hematological malignancies, the differential diagnosis relies on morphological features, certain antigen profiles, and characteristic chromosomal abnormalities.¹³ The most prevalent and diagnostic chromosomal changes in B-cell lymphomas are translocations juxtaposing oncogenes next to the immunoglobulin heavy chain locus (*IGH*) in 14q32.^{14,15} Particular translocations are associated with lymphoma subtype and clinical prognosis: For example, the t(14;18)(q32;q21) involving the *BCL2* gene can be detected in 90% of follicular lymphomas showing an indolent course with a 5-year overall survival of 72%. In contrast, the t(11;14)(q13;q32) is the hallmark of mantle cell lymphoma which is a rapidly progressing disease with a 5-year overall survival of only 27%.¹⁶ The detection of chromosomal aberrations involving the *MYC* locus in 8q24, eg, t(8;14)(q24;q32), is the gold standard for the diagnosis of a Burkitt's lymphoma.¹³ Structural abnormalities in the *BCL6* locus in 3q27, such as t(3;14)(q27;q32), have been found in 40% of diffuse large B-cell lymphomas and might indicate a favorable prognosis.¹⁷

With regard to ALCL, which accounts for 30 to 40% of all pediatric lymphomas, a subset with a better prognosis has been recently identified.¹⁸ In this subset, fusion of part of the *ALK* gene mostly with *NPM* because of a t(2;5)(p23;q35) but also with various other partner genes such as *AT1C* results in aberrant expression of the ALK-tyrosine kinase.¹⁹

As a prerequisite for M-FISH, multicolor interphase FISH (MI-FISH) assays were developed for B-NHLs and ALCLs and validated in cytogenetically well-characterized lymphomas. Thereafter, these MI-FISH assays were combined with specific antibodies to establish the M-FISH technique. M-FISH experiments on B-NHL and ALCL cases were evaluated by automatic image capturing of multiple focal planes, providing a first step toward complete automation of the M-FISH analyses,

including automatic cell detection and multicolor spot counting.²⁰

Materials and Methods

Tumor and Control Samples

Tumor samples for genetic analysis as part of the diagnostic workup were obtained with informed consent from the patients. For MI-FISH experiments, cells from cases with t(8;14), t(11;14), and t(14;18) translocations left from routine tumor cytogenetic analyses were studied. Cells from healthy donors with normal karyotype were used as negative controls. For M-FISH studies, isolated cells obtained from fresh biopsies of patients affected with B-NHL and ALCL and from one ALCL cell line (L82)²¹ (Tables 1 and 2) were resuspended in phosphate-buffered saline to a final concentration of 1×10^4 cells/ml. Two hundred μ l of the cell suspension were spun in a cytocentrifuge (Cytospin 2; Shandon, Frankfurt, Germany) for 5 minutes at 800 rpm, air-dried overnight, and stored at -80°C until use.

Probe Selection

Considering the genetic features of B-NHLs and ALCLs, gene-specific probes to detect the most frequent primary changes in these lymphomas were selected. For B-NHLs, probes spanning the translocation-associated breakpoints in *IGH*, *BCL1*, *BCL2*, *BCL6*,²² and *MYC*^{23,24} were used whereas for ALCLs, a probe flanking the *ALK* gene and probes telomeric to *NPM*²⁵ and *AT1C*²⁶ were applied. These probe sets included commercial, previously published, and self-designed probes. In the latter category, a probe for the *BCL2* gene was designed searching the genome sequence database using the BLAST algorithm at National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast>) with sequences from the MBR and FVT1 breakpoint regions. Moreover, the physical maps deposited in the HUMANace database at the Washington University (<http://genome.wustl.edu/gsc>) were analyzed for appropriate clones.

Probe Preparation and MI-FISH

Cosmids, BACs, YACs, and commercially available probes (LSI *BCL1/IGH*, LSI *ALK*; Vysis, Downers Grove, IL) were used to design the multicolor assays. Table 3 provides a list with the probes and labeling schemes used. Figure 1, a to d and k to n, provide schematic representations of the probes in both metaphase and interphase cells. Clones were grown overnight in the appropriate selective media and DNA was isolated by the Perfectprep Plasmid Maxi kit (Eppendorf, Köln, Germany) from bacterial clones and the Nucleon M1Y kit (Amersham Pharmacia, Freiburg, Germany) from YAC clones. ALU-PCR from yeast DNA was performed as described elsewhere.²⁷ DNA from cosmids or BACs and ALU-PCR products from YACs was labeled with dUTP-DEAC (NEN, Zaventem, Belgium), dUTP-Spectrum Green, dUTP-Spectrum Orange (Vysis), dUTP-Texas Red (Molecular Probes, Leiden, The Nether-

Table 1. Patient Features and Results Derived from the M-FICTION Assay for B-NHL

Case number	Diagnosis	Gender	Age	Sample	Karyotype of the tumor clone	FICTION result
1	MCL	M	57	LN	45,XY,der(4)t(4;11)(q22~24;q24),-6,der(11)t(11;14)(q13;q32),der(14)t(11;14)(q13;q32)t(4;11)(q22~24;q24),add(14)(q21),der(15)t(3;15)(q13;q23),der(?)t(?;1)(?;p2?1)t(?;10)(;q21)	CD20+ cells: nuc ish 3q27(BCL6x2), 8q24(MYCx2), 11q13(BCL1x3), 14q32(IGHx3), 18q21(BCL2x2)(BCL1 con IGHx2)
2	BL	M	12	LN	47,XY,+der(1;7)(p10;p10),t(8;14)(q24;q32)	CD20- /CD20+ cells: nuc ish 3q27(BCL6x2), 8q24(MYCx2), 11q13(BCL1x2), 14q32(IGHx2), 18q21(BCL2x2)
3	BL	M	6	LN	46,XY,t(8;14)(q24;q32)	CD20+ cells: nuc ish 3q27(BCL6x2), 8q24(MYCx3), 11q13(BCL1x2), 14q32(IGHx3), 18q21(BCL2x2)(MYC con IGHx2)
4	BL	M	12	LN	46,t(X;14)(p11;q32),Y,t(8;14)(q24;q32)	CD20- /CD20+ cells: nuc ish 3q27(BCL6x2), 8q24(MYCx2), 11q13(BCL1x2), 14q32(IGHx2), 18q21(BCL2x2)
5	FL	M	35	LN	46,XY,t(14;18)(q32;q21)	CD20+ cells: nuc ish 3q27(BCL6x2), 8q24(MYCx2), 11q13(BCL1x2), 14q32(IGHx3), 18q21(BCL2x3)(IGH con BCL2x2)
6	FL	M	65	LN	46,XY,del(7)(q?22q?31),t(14;18)(q32;q21)	CD20- /CD20+ cells: nuc ish 3q27(BCL6x2), 8q24(MYCx2), 11q13(BCL1x2), 14q32(IGHx2), 18q21(BCL2x2)

MCL, mantle cell lymphoma; BL, Burkitt lymphoma/leukemia; FL, follicular lymphoma; M, male; LN, lymph node.

lands), or dUTP-Cy5 (Amersham Pharmacia) by random priming using the Bioprime kit (Gibco/Life Technologies, Eggenstein, Germany) exchanging the dNTP-Bio with a dNTP mixture containing dATP (1 mmol/L), dCTP (1 mmol/L), dGTP (1 mmol/L), dTTP (0.5 mmol/L), and dUTP-fluor (0.5 mmol/L). Labeled products were purified with Sephadex G-50 columns. Two hundred ng of each probe were co-precipitated for each assay with 5 μ l of Cot1-DNA (Gibco/Life Technologies) and resuspended in 10 μ l of master mix containing 2 $\times$ standard saline citrate, 50% deionized formamide, and 10% dextran sulfate. FISH was performed as described elsewhere.²⁸

M-FICTION

Cytospin slides were thawed and dried for 30 minutes at room temperature, fixed in acetone for 10 minutes, and air-dried. Slides were incubated for 30 minutes at room temperature with primary mAb [anti-CD20 (1:50) for the B-NHL M-FICTION assay or anti-ALK (1:25) for the

ALKoma M-FICTION assay (DAKO, Hamburg, Germany)] diluted in PNM buffer. Fluorescence detection of the primary antibody was performed with a cascade of three sequential antibodies conjugated with amino methylcoumarin (AMCA) that were diluted in PNM buffer²⁸ (1:20) and incubated for 30 minutes each at room temperature: 1) AMCA-conjugated rabbit anti-mouse, 2) AMCA-conjugated goat anti-rabbit, and 3) AMCA-conjugated donkey anti-goat (Jackson ImmunoResearch/Dianova, Hamburg, Germany). After immunophenotyping, slides were fixed in Carnoy's fixative (ethanol:acetic acid, 3:1) for 10 minutes and in paraformaldehyde solution (1%) for 1 minute. Then, slides were dehydrated through increasing ethanol concentrations (70%, 85%, and absolute) and air-dried. The appropriate MI-FISH probe (1.5 μ l) (B-NHL or ALLC multicolor assays) was applied to the cell-containing area of the slide and covered with a round 10-mm coverslip. Both probe and target DNA were simultaneously denatured at 70°C for 12 minutes and incubated overnight at

Table 2. Patient Features and Results Derived from the M-FICTION Assay for ALCL

Case number	Diagnosis	Gender	Age	Sample	Karyotype of the tumor clone	FICTION result
7	ALCL			Cell line L82	70<3n>,add(X)(p22),der(X)t(X;22)(p21;q11),t(2;5)(p23;q35),+5,der(5)add(5)(p14)add(5)(q31),add(5)(q31),add(6)(p24)x2,+7,add(7)(q22),+8, dup(8)(q24q23)x2,add(9)(q11),del(10)(p12),+13,der(13;15)(q10;q10),add(13)(q21),-14,-15,der(16) t(9;16)(q13;p12),-17,-19,add(21)(p13),+2mar	Hyperploid cells with several <i>ALK</i> splits and <i>NPM/ALK</i> fusions. Complex hybridization pattern. The great majority of the cells were positive for the <i>ALK</i> immunostaining.
8	ALCL	M	54	LN	46,Y,t(X;2;5)(q?26;p23;q35) / 47,idem,+i(6)(p10) / 82~92,idem	ALK+ cells: nuc ish 2p23(ALK-Cx2,ALK-Tx2),2q35(ATIC-Tx2), 5q35(NPM-Tx2) (ALK-C sep ALK-Tx1) ALK- cells: nuc ish 2p23(ALK-Cx2,ALK-Tx2),2q35(ATIC-Tx2),5q35(NPM-Tx2)
9	ALCL	M	6	LN	50~51,XY,+X,del(1)(q21),+2,der(2)?dup(2)(p25p21)dup(2)(p11p25)x2,+17	ALK+ cells: nuc ish 2p23(ALK-Cx3,ALK-Tx3),2q35(ATIC-Tx3),5q35(NPM-Tx3)(ALK-C sep ALK-Tx2) ALK- cells: nuc ish 2p23(ALK-Cx2,ALK-Tx2),2q35(ATIC-Tx2), 5q35(NPM-Tx2)
10	ALCL	M	6	LN	47,XY,t(2;5)(p23;q35),+7 / 47,idem,t(5;13)(q13;q14)	ALK+ cells: nuc ish 2p23(ALK-Cx2,ALK-Tx2),2q35(ATIC-Tx2),5q35(NPM-Tx2) (ALK-C sep ALK-Tx1) (ALK-C con NPM-Tx1) ALK- cells: nuc ish 2p23(ALK-Cx2,ALK-Tx2),2q35(ATIC-Tx2),5q35(NPM-Tx2)

ALCL, anaplastic large-cell lymphoma; M, male; LN, lymph node.

37°C. Posthybridization washes were performed in 0.1× standard saline citrate three times for 5 minutes each at 60°C. Then, slides were washed once in PN²⁸ buffer and mounted in anti-fade solution. No DNA counterstaining was performed.

Image Acquisition and Processing

Slides were analyzed by using a motorized epifluorescence microscope (Axioplan 2 imaging mot, Zeiss) equipped with the appropriate filter sets and the MetaSystems isis/mFISH

Table 3. DNA Probes and Labeling Schemes Applied in the Present Study

Locus	Clone	Dye	Location to breakpoint	References
B-cell non-Hodgkin's lymphomas (B-NHL)				
<i>IGH</i> (14q32)	No data	SG	Spanning	Vysis, Downers Grove, IL (http://www.vysis.com)
<i>BCL1</i> (11q13)	No data	SO	Spanning	Vysis, Downers Grove, IL (http://www.vysis.com)
<i>BCL2</i> (18q21)	b495C15 b299P2 b718L4	DEAC	Spanning MBR and FVTI breaks	
<i>BCL6</i> (3q27)	b528E8 b690C8	TR	Spanning	22
<i>MYC</i> (8q24)	cMYC72 cH4.1 cP380J9 y904C3 y934E1	Cy5	Spanning	23 24
Anaplastic large cell lymphomas (ALCL)				
<i>ALK</i> (2p23)	No data	SG	Centromeric	Vysis, Downers Grove, IL (http://www.vysis.com)
<i>NPM</i> (5q35)	y939F4	TR	Telomeric	25
<i>ATIC</i> (2q35)	y770F5	Cy5	Telomeric	26

b, BAC clone; c, cosmid; y, YAC clone; SG, spectrum green; SO, spectrum orange; DEAC, diethyl aminomethyl coumarin; TR, Texas Red; Cy5, cyanine 5.

Figure 1. a–j: Composite picture of the MI-FISH and M-FICTION assays for B-NHLs. MI-FISH and M-FICTION images are shown using a false color display. **a:** Ideograms of chromosomes 3, 8, 11, 14, and 18 showing the *BCL6* (3q27), *MYC* (8q24), *BCL1* (11q13), *IGH* (14q32), and *BCL2* (18q21) probes labeled with different dyes. **b:** Schematic representation of an interphase nucleus containing two isolated copies for each of the genes studied. **c:** Ideograms of the same chromosomes but with a t(8;14)(q24;q32) translocation. Derivative chromosomes 8 and 14 show co-localization of green and orange signals. **d:** Schematic representation of an interphase nucleus in which the t(8;14)(q24;q32) translocation is observed as double split of green and orange signals (three signals of each color) and double co-localization of green and orange signals. **e–h:** MI-FISH in negative and positive controls using the B-NHL multicolor probe set. DAPI is used as nuclear counterstain. **e:** Intact signal constellation displaying two signals for each locus. **f–h:** MI-FISH on B-cell lymphomas with *IGH* translocations affecting *MYC*, *BCL1*, and *BCL2* are shown, respectively. Notice that a translocation event leads to double split and double co-localization of the signals from the genes involved in the translocation. **g:** Four co-localizations, reflecting that the captured cell had additional der(11) or der(14) as well as additional signals for *BCL6* that might indicate polyploidy. **i–j:** M-FICTION in a t(8;14)-positive Burkitt's lymphoma (case 3) using a mAb anti-CD20 and the B-NHL multicolor probe set. **i:** CD20-negative cell showing a normal pattern of hybridization signals. The blue background staining derives from artificial enhancement necessary to visualize the cell shape. As intact cells were used for the M-FICTION analyses, a pseudo-nuclear CD20 staining would be expected. The lack of such nuclear staining clearly indicates CD20 negativity. **j:** CD20-positive (intense blue staining) cell displaying a double split and double co-localization of the *MYC* (orange) and *IGH* (green) signals, which indicates the presence of a t(8;14). That translocation was detected exclusively in the CD20-positive B-cell subset. **k–p:** Composite picture of the M-FICTION assay for ALCLs. M-FICTION images are shown using a false color display. **k:** Ideograms of chromosomes 2 and 5 showing the *ALK* (2p23), *ATIC* (2q35), and *NPM* (5q35) differentially labeled probes. **l:** Ideograms of the same chromosomes but with a t(2;5)(p23;q35). Derivative chromosome 2 shows a green/pink co-localization and derivative 5 shows an isolated red signal. **m:** Schematic representation of an ALK-negative interphase cell (gray) containing two co-localizations for *ALK* (green centromeric/red telomeric) and two isolated signals for the telomeric part of the *NPM* (pink) and *ATIC* (orange) breakpoint regions. **n:** Schematic representation of an ALK-positive interphase cell (blue indicates AMCA) in which the t(2;5)(p23;q35) translocation leads to the dissociation of the green and red probes flanking the *ALK* gene and furthermore to the co-localization of the *ALK* centromeric probe (green) with the probe telomeric to the breakpoint in the *NPM* gene (pink). This co-localization takes place in the der(2) derivative chromosome and an isolated red signal (*ALK* telomeric) is located in the der(5). **o:** M-FICTION in a patient with a classical t(2;5) translocation (patient 10). One immunophenotypically positive cell for ALK and two negative cells are shown. The two small ALK-negative (without blue staining) of the upper part of the picture display a normal pattern of hybridization signals: two signals for each probe and co-localization of green and red signals derived from probes flanking the *ALK* gene (arrowheads). The large ALK-positive (blue staining) cell has a signal pattern indicating the presence of a t(2;5)(p23;q35) translocation. A split of one green and one red signal indicates the break within the *ALK* locus. The arrows point to a green/pink co-localization derived from the fusion of the *ALK* and *NPM* genes. This translocation was found exclusively in those cells expressing ALK. The co-localization of the *ALK*-tel and *ATIC*-tel signal (red and orange signals, respectively) was random in this cell and is not indicative for an *ATIC/ALK* fusion that would result in co-localization of the *ATIC*-tel (orange) signal with the *ALK*-cen (green) signal. The arrowhead points to an unrearranged *ALK* allele, shown as co-localization of red and green signals. **p:** M-FICTION in patient 9 showing an ALK-positive cell. The arrowheads point to rearrangements of the *ALK* gene—displayed as split of red and green probes—without co-localization with *NPM* or *ATIC*. The arrowhead points to an unrearranged *ALK* allele. The co-localization of one green and one orange signal at the top of the figure is because of random overlapping, because it did not appear in the other cells expressing ALK. All of the cells in this patient displayed two couples of isolated red and green signals indicating two rearranged *ALK* loci. This case was previously shown as bearing an *ALK/TPM3* fusion.³⁰

imaging system (MetaSystems, Altussheim, Germany). 4,6-Diamidino-2-phenylindole (DAPI) (for MI-FISH) or AMCA (for M-FICTION) images were captured with a DAPI filter set, whereas for the DEAC, SG, SO, TR, and Cy5 fluorochromes narrow band-pass filter sets were used. For both MI-FISH and M-FICTION, images from five different focus planes separated by a distance of 1 μm were captured automatically. Then, a composite image was calculated, leading to transformation of the three-dimensional information into a two-dimensional picture containing all of the hybridization signals.

Results

Technical Considerations: Labeling and Digital Imaging

Multicolor cytogenetic studies require the application of multiple differentially labeled probes, that can be achieved by using several fluorescence dyes and/or by combinatorial labeling schemes.^{10,11} Our MI-FISH and M-FICTION assays aimed to obtain the maximum diagnostic information applying a minimum number of probes. Thus, the probe design allowed us to label each probe with a single dye rather than using combinatorial labeling, rendering image processing and routine evaluation of the hybridization signals easier.

We performed image acquisition by using the widely available filter-based technology that applies narrow band-pass filters specific for single fluorochromes.¹¹ Capturing several signals in a two-dimensional picture poses the problem that signals are distributed within a three-dimensional structure (the cell nucleus), and some signals might be in different focus planes. The use of the mFISH software package from MetaSystems (Altussheim, Germany) and a Z axis motorized microscope (Zeiss, Göttingen, Germany) allowed several focus planes to be captured and reconstructed into a single image. A final multicolor picture containing all of the hybridization signals was achieved by merging the three-dimensional information derived from each color channel.

Establishment of M-FICTION Assays for Hematological Neoplasms

B-NHLs

This multicolor assay designed with probes for *IGH* (SG), *BCL1* (SO), *BCL2* (DEAC), *BCL6* (TR), and *MYC* (Cy5) does not only target the diagnostically and prognostically most important primary aberrations in B-cell lymphomas but should also be able to detect at least one genetic marker in up to 70% of B-NHL (Figure 1; a to d).²⁹ The reliability of the multicolor locus-specific probe set to detect B-NHL-associated translocations was assessed by MI-FISH in negative and positive controls (Figure 1; e to h). We applied these probes in combination with a mAb anti-CD20 to lymph node specimens from six cytogenetically characterized cases of primary B-NHL. In all cases, we could reliably detect the diagnostic transloca-

tions, eg, t(11;14), t(8;14), or t(14;18), within the CD20-positive B-cell compartment (Table 1 and Figure 1, i and j). In every case, nonmalignant CD20-positive and -negative cells (with normal multicolor signal constellations) were also observed, representing the normal B-cell and non-B-cell infiltrate in the tumor tissue. In case 4, an additional green signal (*IGH* locus) was found within the CD20+ subset, which is most likely because of a split of the *IGH* probe derived from the t(X;14)(p11;q32) translocation appearing in the same tumor clone as the t(8;14) (Table 1).

ALCLs

This assay was designed with differentially labeled probes for *ALK* (centromeric, SG; telomeric, SO), *NPM* (telomeric, TR), and *AT1C* (telomeric, Cy5) (Figure 1; k to n). The reliability of this multicolor probe set was confirmed by MI-FISH in negative and positive controls (data not shown). By means of the M-FICTION assay, that combines the ALCL-specific multicolor probe set with a mAb anti-ALK, we identified the aberrant expression of ALK along with the characteristic genetic changes in a cell line and three primary cases of ALKomas with *NPM/ALK* ($n = 3$), two with a t(2;5) and one with a t(X;2;5) translocation, and one with *TPM3/ALK* fusion (Table 2). However, as we used cytopspins from fresh material, in which nuclei are overlaid by cytoplasm, we could not reliably define the expression pattern of ALK (cytoplasmic versus cytoplasmic and nuclear). Because of the tumor cell specificity of the ALK staining, the cells from primary cases expressing ALK displayed signal constellations indicating rearrangements within the *ALK* gene whereas the signal patterns in ALK-negative cells lacked evidence for such a change (Figure 1, o and p). In cases 8 and 9, split of the probe flanking the *ALK* locus without co-localization with *NPM* or *AT1C* was observed, pointing to variant rearrangements of the *ALK* locus (Figure 1p). Case 8 carries a cytogenetically proven t(X;2;5) translocation. In line with this finding, M-FICTION failed to identify a fusion of *ALK-C* with the telomeric part of *NPM*. A translocation of the distal part of *NPM* to an unknown region in Xq26 would be expected in this case. Case 9 showed a cytogenetically unresolved derivative chromosome 2 described as der(2)?dup(2)(p25p21)dup(2)(p11p25). This case has previously been shown to contain a complex rearrangement leading to a *ALK/TPM3* fusion.³⁰ In accordance with this finding, M-FICTION indicated a variant ALK rearrangement neither affecting *NPM* nor *AT1C*.

Discussion

The newly developed M-FICTION technique provides a new tool for the simultaneous and comprehensive characterization of morphological, immunophenotypic, and genetic features of single cells. We tested the performance of the technique by developing assays for the diagnosis of lymphoid malignancies.¹³ The M-FICTION assays designed for B-NHL and ALCL combine the detection of specific anti-

gens with probes for the most characteristic chromosomal aberrations in these neoplasms. To resolve a given diagnostic situation, M-FICTION assays can be customized at will by exchanging probes and antibodies. For instance, it is possible to combine probes for all three *IG* loci³¹ along with a probe for the *MYC* locus to detect classical and variant Burkitt translocations. With regard to expenses, we estimate that the materials for one M-FICTION hybridization as described herein would cost ~\$15.

The application of the M-FICTION technique is certainly not limited to hematological neoplasms. Fields of future applications are, for example, the detection of micrometastasis of solid tumors or of disseminated tumor cells in bone marrow or peripheral blood.^{32,33} Moreover, contaminating tumor cells in stem cell transplants or minimal residual disease after treatment could be detected by M-FICTION. Additionally, M-FICTION can visualize aberrations at gene and protein level simultaneously, which might prove clinically important in some cases of breast or pancreatic cancer in which conflicting results between immunohistochemistry and FISH were obtained for *HER2/NEU* gene amplification and increased gene expression.³⁴ M-FICTION assays could also be used for the early detection of cancer. As an example, the early detection of bladder cancer cells in urine might be improved by an M-FICTION assay combining commercially available assays relying on immunohistochemistry and FISH.^{35,36} Finally, M-FICTION assays might also be applied to basic research in experimental cell biology aiming for example, to unravel the mechanisms of recombination³⁷ or to study interactions between DNA and proteins³⁸ such as between chromosomes and centrosomes.³⁹

In the present study, M-FICTION was restricted to the investigation of a single antigen in combination with up to five FISH probes, which were sufficient to detect the diagnostically most important chromosomal aberrations in the tumors investigated. Likewise, the UroVysion and LAVysion FISH tests (Vysis) detect a genetic marker in ~80% of bladder cancers³⁶ and lung cancers, respectively, by means of only four probes. To increase the number of FISH probes—a maximum of five in this study—new dyes as well as combinatorial labeling could be applied. Furthermore, as we applied a miniaturized hybridization format,⁴⁰ it is possible to perform up to 12 MI-FISH or M-FICTION assays on a single slide and therefore, a single slide could be hybridized with 60 different probes (5 probes × 12 hybridization areas). Moreover, M-FICTION might not be restricted to fresh samples. If sufficient antigen retrieval and DNA accessibility can be achieved, the M-FICTION technique is in principle also applicable to paraffin-embedded sections, including tissue CHiPs.⁴¹

In the future, the importance of M-FICTION for the diagnosis and research of malignant tumors might take significant advantage from the achievements of high-throughput expression studies using, for example, CHIP technology. This approach aims to identify mRNAs and proteins differentially expressed in biological and prognostic subgroups of malignancies.⁴² By means of this technology, the expression of a few genes has been recently significantly correlated either with refractory/fatal

or cured diffuse large B-cell lymphoma patients.⁴³ In CLL, recent microarray studies have shown a restricted number of genes to distinguish between IgV-mutated and -unmutated cases,^{44,45} having the latter group a distinctly worse prognosis than the former.⁴⁶ By M-FICTION, the diagnostic and prognostic genetic aberrations and the discriminating proteins identified in these and upcoming expression studies could be sensitively and simultaneously detected at the single cell level.

The multiple applications of M-FICTION and its suitability for automation, miniaturization, and high-throughput analysis might render this technique a valuable tool not only for the characterization of malignant tumors but also for many other fields of medical research and experimental cell biology.

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