

Detection of Chromosome Abnormalities in Leukemia Using Fluorescence *In Situ* Hybridization

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1. Introduction

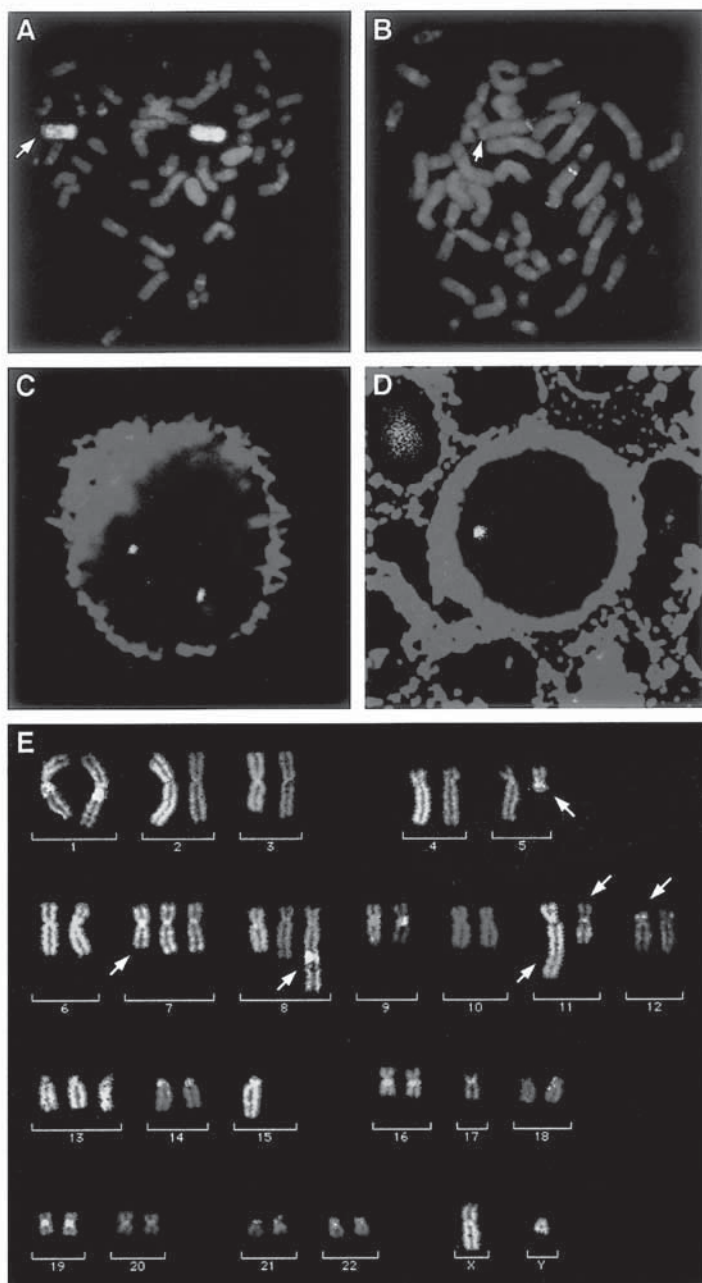
Cytogenetic analysis plays a pivotal role in the diagnosis and management of patients with hematologic malignancies. In research, the identification of specific chromosomal rearrangements associated with defined clinical groups has led to an explosion in the knowledge of basic mechanisms contributing to leukemogenesis. The strength of cytogenetic analysis is as a direct method for screening the whole genome. However, the interpretation of the banding pattern of highly rearranged chromosomes is often unreliable. Since the advent of molecular cytogenetic technologies based around fluorescence *in situ* hybridization (FISH), the accuracy of cytogenetic diagnosis has been considerably enhanced. Specific problems hampering the accurate analysis of leukemic karyotypes such as the low mitotic index, heterogeneity of the sample, and often poor morphology of chromosomes are also largely overcome by FISH. One of the most significant advances is the use of interphase FISH, which permits the use of nondividing cells as DNA targets and enables a large number of cells to be evaluated (1–4). This has advantages for monitoring disease progression, response to treatment, and success of bone marrow transplantation. The simultaneous identification of cell type (by morphology or immunophenotype) and chromosome abnormality (by FISH) is also possible, allowing the identification of cell lineages involved in the neoplastic clone (5).

The application of FISH to metaphase chromosomes provides unequivocal evidence of chromosome rearrangements. Whole-chromosome painting probes, derived from chromosome-specific libraries, or polymerase chain reaction (PCR) amplification of flow-sorted or microdissected chromosomes can be used to identify accurately the components of complex rearrangements and marker chromosomes (6–10). Chromosome-specific centromeric probes,

targeting the tandemly repeated alpha (or beta) satellite sequences present in the heterochromatin of chromosome centromeres, are invaluable for the rapid visualization of numerical chromosome abnormalities. Specific gene probes for the detection of leukemia-associated translocations and inversions (**11–13**) allow accurate detection of these rearrangements, especially in complex or masked versions of the translocation, and are particularly useful for interphase analysis. A significant advance in the resolution of FISH for the visualization of translocations is provided by hybridization to extended DNA fibers, so-called fiber-FISH. This is particularly valuable for the analysis of chromosome rearrangements with highly variable breakpoints, provided there is a well-characterized contig of the region (**14,15**).

One of the most appealing aspects of FISH is the ability to identify several targets simultaneously using different colors (so-called multicolor FISH) (*see Fig. 1A*). The most recent developments in this area are those that enable “color karyotyping,” using whole-chromosome painting probes that delineate each of the 22 pairs of autosomes and the sex chromosomes in a different color. The related techniques of multiplex-FISH (M-FISH) and spectral karyotyping (SKY) (**16,17**) provide the prospect of a molecular analysis of karyotype. One of these, SKY, is detailed in Chapter 3. Herein we outline the basic FISH methodologies, as well as some of the more advanced techniques, with particular reference to specific applications in hematologic malignancy. Further specialized *in situ* hybridization methods are given in **ref. 18**.

Fig. 1. Examples of applications for FISH in leukemia. (**A**) Dual-color FISH using whole chromosome painting probes for chromosomes 7 (green) and 12 (red) to a leukemic metaphase from an acute myeloid leukemia patient. This identified a cryptic translocation between chromosomes 7 and 12. In this metaphase chromosome 12 material is visible on the der(7) (arrow); however, the reciprocal chromosome 7 material is not visible on the der(12). (**B**) Dual-color FISH to map the extent of the 5q deletion in a leukemic metaphase from a patient with 5q- syndrome. A 5p subtelomeric probe (red) was cohybridized to tag both chromosomes 5, to ensure that only metaphases containing the del(5q) were evaluated. Green fluorescent signal corresponding to a probe containing the *CSF1R* gene is present on the normal homolog, but absent from the del(5q) (arrow). (**C,D**) Combined immunophenotyping and FISH (using a YAC probe containing the *CSF1R* gene) to bone marrow cells from a patient with a del(5q) clonal chromosome marker. In each case, the APAAP positive cells show bright red fluorescence. In (C) the CD3 antibody identifies a T cell with two green fluorescent FISH signals corresponding to the YAC. In (D) the glycophorin A antibody identifies an erythroid precursor with only one fluorescent signal. This confirms that the erythroid, but not the T-lymphocyte, lineage is involved in the malignant



clone in this patient. (E) M-FISH karyotype of a metaphase from the leukemia-derived cell line GF-D8. Metaphases were hybridized with a set of combinatorially labeled whole chromosome painting probes, and chromosomes assigned a pseudocolor according to their unique fluorochrome composition using Powergene M-FISH software (Applied Imaging, Newcastle, UK). Structurally abnormal chromosomes thus identified are indicated by arrows.

2. Materials

2.1. Preparation of Bone Marrow Metaphase Chromosomes

1. Bone marrow aspirate collected into sterile bottles containing transport medium (RPMI 1640 plus 50 U/mL of penicillin, 50 μ g/mL of streptomycin, and 10 U/mL of preservative-free lithium heparin).
2. Thymidine, crystalline (Sigma, St. Louis, MO): 100 μ M stock.
3. 5-Fluorodeoxyuridine (Sigma): 100 μ M stock.
4. Uridine (Sigma): 400 μ M stock.
5. Colcemid (10 μ g/mL) (Gibco).
6. Culture medium: RPMI 1640, 50 U/mL of penicillin, 50 μ g/mL of streptomycin, 2 mM L-glutamine, 20% fetal calf serum (FCS) (all from Gibco-BRL).
7. Hypotonic solution: 0.075 M KCl.
8. Fixative: 3:1 AnalaR methanol:glacial acetic acid, at 4°C.
9. Precleaned microscope slides (Superfrost, BDH).

2.2. Pretreatment of Chromosomes and Nuclei

1. Pepsin (100 mg/mL) (Sigma).
2. Phosphate-buffered saline (PBS)/50 mM MgCl_2 : 50 mL of 1 M MgCl_2 + 950 mL of 1X PBS.
3. PBS/50 mM MgCl_2 /1% formaldehyde (make up fresh each time): 2.7 mL of formaldehyde in 100 mL of PBS/ MgCl_2 .
4. PBS (1X): 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 , 0.2 g of KH_2PO_4 in 800 mL of H_2O , pH to 7.4 with HCl. Add H_2O to 1 L.
5. RNase A (10 mg/mL) (Sigma) (boiled for 10 min to remove contaminating DNase).
6. Formaldehyde (40% [w/v]).

2.3. Preparation of Probe DNA

2.3.1. Cosmids, P1 Artificial Chromosomes (PACs)

1. 2X TY medium (1 L): 16 g of Bacto tryptone, 10 g of yeast extract, 5 g of NaCl.
2. Glucose/EDTA/Tris (GET): 0.9% glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 7.0.
3. NaOH/sodium dodecyl sulfate (SDS): 0.2 M NaOH, 1% SDS.
4. 3 M KOAc, pH 5.5.
5. RNase A (DNase free) (10 mg/mL) (Sigma).

2.3.2. Yeast Artificial Chromosomes (YACs)

1. YEPD medium (1 L): 10 g of Bacto yeast extract, 20 g of Bactopeptone, 20 g of dextrose, 10 mL of adenine sulfate (0.5% in 0.5 M of HCl).
2. GDIS: 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA.
3. Phenol:chloroform:isoamyl alcohol (25:24:1).
4. RNase A (DNase free) (10 mg/mL) (Sigma).
5. Glass beads, 710–1180 μ m, acid washed (Sigma).

2.4. Nick Translation Labeling

1. Purified probe DNA (1 μ g).
2. 10X Nick translation buffer: 0.5 M Tris-HCl, pH 7.5, 50 mM $MgCl_2$, 0.5 mg/mL of nuclease-free bovine serum albumin (BSA).
3. 1 mM Biotin-16-dUTP (bio-16-dUTP), 1 mM digoxigenin-11-dUTP (dig-11-dUTP) (Roche Diagnostics).
4. 100 mM Dithiothreitol (DTT) (Sigma).
5. dNTP mix: 0.5 mM each dATP, dCTP, dGTP, and 0.1 mM dTTP (Roche Diagnostics).
6. DNase 1 (200,000 U) (Roche Diagnostics).
7. DNase 1 dilution buffer: 50% glycerol, 0.15 M NaCl, 20 mM sodium acetate, pH 5.0.
8. DNA polymerase 1 (10 U/ μ L) (New England Biolabs).
9. MicroSpin G50 columns (Amersham Pharmacia Biotech).
10. *Escherichia coli* tRNA (10 mg/mL) (Roche Diagnostics).
11. Salmon sperm DNA (5 mg/mL, sonicated to 200–500 bp) (Sigma).
12. TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.
13. Gel-loading buffer (5X bromophenol blue): 10% (w/v) Ficoll, 0.1 M Na_2 EDTA, 0.5% (w/v) SDS, 0.1% (w/v) bromophenol blue.
14. Electrophoresis buffer (10X TBE): 108 g of Tris base (89 mM), 55 g of boric acid (89 mM), 40 mL of 0.5 M EDTA, pH 8.0 (2 mM) per liter.
15. *PhiX174 HaeIII* size marker (BRL Life Technologies).

2.5. Competitive In Situ Suppression Hybridization

1. Human Cot-1 DNA (BRL Life Technologies).
2. 3 M Sodium acetate.
3. Denaturing solution: 70% (v/v) formamide, 2X saline sodium citrate (SSC), 0.1 mM EDTA, pH 7.0.
4. Hybridization buffer: 50% (v/v) formamide, 10% (w/v) dextran sulfate, 1% (v/v) Triton X-100, 2X SSC, pH 7.0.
5. Formamide (purified) (Fluka).
6. 50% Dextran sulfate.
7. 20X SSC: 1X SSC = 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0.
8. Blocking solution: 3% (w/v) BSA in 4X SSC, 0.05% (v/v) Triton X-100 (make up fresh).
9. Wash solution: 4X SSC, 0.05% (v/v) Triton X-100.

2.6. Detection of Bound, Labeled Probe

1. Fluorescence microscope (epifluorescence illumination), with suitable fluorescence objectives and filter sets (usually need separate filter sets for fluorescein isothiocyanate [FITC], Texas red/rhodamine and 4,6-diamidino-2-phenylindole [DAPI]/AMCA, as well as a double or triple filter block).
2. Avidin-DCS-FITC (1 mg/mL) (Vector).
3. Biotinylated anti-avidin D (0.5 mg/mL) (Vector).
4. Propidium iodide (Sigma).

5. DAPI (Sigma).
6. Vectashield mountant (Vector).
7. Avidin DCS-Texas red (2.5 mg/mL stock) (Vector).
8. Diluent for antibodies: blocking solution, filtered through a 0.45- μ m syringe filter. Stock antibody solutions are stored at -20°C .
9. Monoclonal antidigoxigenin (Sigma).
10. Rabbit antimouse Ig-FITC (Sigma).
11. Monoclonal antirabbit-FITC (Sigma).

2.7. Degenerate Oligonucleotide Primer-PCR Amplification of Flow-Sorted Chromosomes

1. Flow-sorted chromosomes (approximate concentration: 500/ μL).
2. 2X PCR buffer: 10 mM MgCl_2 , 100 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mg/mL of gelatin.
3. dNTP mix: 2 mM each dATP, dCTP, dGTP, dTTP.
4. 6-MW primer: 5' CCGACTCGAGNNNNNNATGTGG 3' (30 μM).
5. *Taq* 1 (2.5 U/ μL) polymerase (Roche Diagnostics).
6. 1 mM Biotin-16-dUTP or 1 mM dig-11-dUTP (Roche Diagnostics).

2.8. Alkaline Phosphatase Antialkaline Phosphatase Staining

1. Thin bone marrow smears (store unfixed wrapped in foil at -20°C).
2. Tris-buffered saline (TBS): 1 M Tris, 0.5 M NaCl.
3. Appropriate primary monoclonal antibody.
4. Rabbit antimouse antibody (Z259; Dako, Cambridge, UK) diluted 1:500 in TBS.
5. Monoclonal alkaline phosphatase antialkaline phosphatase (APAAP) complex (1:500 dilution) (Roche Diagnostics).
6. Alkaline phosphatase substrate: Dissolve 2 mg of naphthol AS mix (Sigma) into 10 mL of 0.1 M Tris buffer (pH 8.2). To this add 10 mg of Fast Red TR mix (Sigma) and dissolve. Then add levamisole (0.1 M) (Sigma) to block endogenous alkaline phosphatase. Filter before use.

3. Methods

3.1. Preparation of Target Material

3.1.1 Culture and Harvesting of Mitotic Chromosomes from Leukemic Bone Marrow (see ref. 19)

1. Set up between one and four cultures, depending on the white cell count. Each culture should contain approx 1×10^6 cells/mL. In most cases, the following will suffice:
 - a. Direct harvesting after 1 h exposed to colcemid (0.1 $\mu\text{g}/\text{mL}$).
 - b. A 24-h incubation with the addition of colcemid for the last hour.
 - c. Twenty-four hour synchronized cultures. For these, add fluorodeoxyuridine (0.1 μM) and uridine (4 μM) after 24 h and reincubate the cultures overnight (16–18 h). Finally, add thymidine (10 μM), and reincubate for 5 to 6 h before adding of colcemid for 10 min before harvesting.

2. Centrifuge at 100g for 5 min. Discard the supernatant and resuspend the pellet in hypotonic solution (prewarmed to 37°C). Incubate at 37°C for 20 min.
3. Centrifuge, discard the supernatant, and mix the pellet in the small volume of hypotonic solution remaining. Add freshly made fixative dropwise, with mixing. Add the first milliliter of fixative slowly, and then make up to 10 mL.
4. Leave in fixative for 30 min at 4°C. Centrifuge at 100g for 5 min, then wash in three to five changes of fixative before making slides.
5. Wipe Superfrost slides clean with absolute ethanol just before use.
6. Place a drop of cell suspension on each slide and air-dry. Monitor the quality of chromosome spreading under phase contrast. Chromosomes should be well spread without visible cytoplasm and should appear dark gray under phase contrast (not black and refractile or light gray and almost invisible).

The “direct” culture can be replaced by overnight incubation with colcemid (0.5 µg/mL). For cell lines, culture according to their specified growth requirements, then harvest when growing logarithmically, usually 24–48 h after a change of medium. Add colcemid for the final 1 h before harvesting.

3.1.2. Preparation of Interphase Nuclei

Interphase nuclei are present in large numbers on slides from leukemic bone marrow or blood prepared as in **Subheading 3.1.1**. Interphase nuclei can also be prepared from fresh bone marrow after Ficoll separation of mononuclear cells. After washing pellets in culture medium (RPMI, without FCS), fix the cell pellet in several changes of methanol:acetic acid (3:1). Drop onto clean slides. Nuclei from a variety of tissues and culture types can be prepared by cytospin, then fixed in methanol (10–20 min). Bone marrow smears are prepared in the usual way and stored unfixed, wrapped in foil at –20°C until required.

3.2. Pretreatment of Chromosomes and Nuclei

The methanol/acetic acid fixation of metaphase chromosomes removes some basic proteins that might interfere with hybridization. However, there is still a variable amount of other protein and cytoplasmic contaminants on metaphase chromosome preparations that may block hybridization, or cause nonspecific background. We routinely use an RNase treatment and postfixation with formaldehyde. For interphase FISH, it may be necessary to add a proteolytic digestion (e.g., pepsin) treatment to this, to aid access of the probe and detection reagents. However, overdigestion can cause loss of cells from slides, so use only when absolutely necessary.

1. Place 100 µL of RNase A (100 µg/mL) on slides under a 24 × 50 mm coverslip and incubate at 37°C for 30 min to 1 h.
2. Wash three times (3 min each) in 2X SSC (with agitation).

3. Pepsin treatment (optional): 50 $\mu\text{g}/\text{mL}$ in 0.01 *M* HCl. Incubate for 10 min at RT.
4. Wash (two times for 5 min each) in 1X PBS.
5. Wash (once for 5 min) in 1X PBS/50 *mM* MgCl_2 .
6. Fix in PBS/50 *mM* MgCl_2 /1% formaldehyde (2.7 mL of formaldehyde in 100 mL of 1X PBS/50 *mM* MgCl_2 [fresh solution]) for 10 min.
7. Wash in 1X PBS for 5 min (with agitation).
8. Dehydrate slides through an alcohol series (70%, 95%, absolute) and allow to air-dry. Slides can be stored desiccated at 4°C for up to 1 mo before use (*see Note 1*).

3.3. Preparation of Probe DNA

3.3.1. Cosmid, P1, and PAC DNA

Any DNA purification method that produces DNA suitable for sequencing will generally also work for FISH. The following medium-scale alkaline lysis method gives a high yield of cosmid, PAC, or P1 DNA. However, this is relatively impure and may require additional purification steps. As a guide, if the DNA fails to cut with DNase I, purify with phenol/chloroform or CsCl gradient centrifugation.

1. Inoculate 250 mL of 2X YT medium plus antibiotic (final concentration: 30 $\mu\text{g}/\text{mL}$ of kanamycin, 50 $\mu\text{g}/\text{mL}$ of ampicillin) in a 500-mL sterile plugged flask with a single well-separated colony.
2. Grow at 37°C with shaking (300 rpm) until approaching saturation (approx 18 h).
3. Transfer to a 250-mL bottle. Centrifuge at 4000g for 10 min.
4. Discard the supernatant medium and drain briefly. Add 50 mL of cold glucose/EDTA/Tris (GET). Resuspend by drawing up in a 10-mL pipet.
5. Add 50 mL of NaOH/SDS at room temperature. Mix by very gentle, minimal inversions. Leave for 5 min (room temperature).
6. Add 50 mL of cold 3 *M* KoAc. Mix by very gentle, minimal inversions. Place on ice for 20 min.
7. Centrifuge at 9000g for 20 min (4°C).
8. Carefully transfer the supernatant to a fresh 250-mL bottle through a mesh.
9. Add 90 mL of isopropanol (0.7X vol) and mix. Leave at room temperature for 5 min.
10. Centrifuge at 5000g for 15 min at room temperature. Discard supernatant.
11. Add 25 mL of 70% ethanol, and rotate the bottle to rinse the inner surface. Transfer pellet to 50-mL Falcon tubes.
12. Centrifuge at 5000g for 5 min (4°C). Discard the supernatant.
13. Allow to stand for 1 min, and then remove final traces of 70% ethanol with a Gilson.
14. Air-dry. Resuspend in approx 200 μL of H_2O (or TE).
15. Incubate with RNase A (final concentration: 30 $\mu\text{g}/\text{mL}$) at 37°C for 30 min.

3.3.2. Yeast Artificial Chromosome DNA (20)

The following method yields high quantities of total yeast DNA suitable for FISH (*see Note 2*). The average yield from a 10-mL culture is 10–20 μg .

1. Culture cells at 30°C for up to 2 d in 10 mL of YEPD medium (grow to saturation).
2. Centrifuge (1500g, 10 min) to pellet the cells, and discard the supernatant. Transfer to an Eppendorf tube, and wash the cells with 500 μ L of distilled water.
3. Centrifuge and then resuspend in 200 μ L of GDIS. Add 0.35 g of glass beads and 200 μ L of phenol. Vortex continuously for 5 min.
4. Add 200 μ L of distilled water to the suspension, mix well, and spin for 4 min in a microcentrifuge.
5. Extract once more with phenol, then once with phenol:chloroform:isoamyl alcohol.
6. Precipitate the DNA as usual (0.1X sodium acetate, 2X absolute ethanol) followed by a 70% ethanol rinse.
7. Remove the aqueous layer and treat this with 50 μ g/mL of RNase A for 20 min at 37°C.
8. Dry the pellet and resuspend in 20 μ L of distilled water.
9. Measure the DNA concentration accurately, preferably in a fluorometer (*see Note 3*).

3.4. Nick Translation Labeling of Probes

Nick translation is the most widely used method for labeling probes for *in situ* hybridization, because the fragment size can be controlled by the amount of DNase I in the reaction mixture. As nick translation is highly efficient for labeling double-stranded circular DNA molecules, there is no need to isolate the insert from the vector sequences. The size of labeled probe fragments is a critical factor in *in situ* hybridization protocols, with an average size of 300 bp being optimal (range 100–500 bp). Larger probe fragments will result in bright background fluorescence all over the slide, obscuring any specific signal. If the labeled probe fragments are too small (<50 bp), the site of hybridization may not be visible owing to the resulting weak fluorescent signal. To ensure the correct size of labeled fragments, it is necessary to run a small aliquot of labeled probe on a 2% agarose gel. Other labeling methods (e.g., random primer labeling, PCR) can be used to produce labeled probes for FISH. However, in all cases the size of the labeled fragments must be checked, and recut with DNase I, if necessary. Probes for localization by FISH are usually labeled with either biotin or digoxigenin, available conjugated to dUTPs by a spacer arm of variable length (e.g., bio-16-dUTP, dig-11-dUTP). Various fluorochromes including FITC, and the cyanine dyes Cy3, and Cy5 are now available directly conjugated to dUTP (Amersham Pharmacia Biotech), enabling direct labeling of DNA.

1. Add the following (in order) to a 1.5-mL Eppendorf tube on ice:
 - a. 1 μ g of probe DNA.
 - b. 1.2 μ L of 1 mM bio-16-dUTP, dig-11-dUTP, or fluorochrome-dUTP.
 - c. 5 μ L of dNTP mix.
 - d. 5 μ L of 10X nick translation buffer.
 - e. 5 μ L of 100 mM DTT.
 - f. Sterile-distilled water to make up to a final volume of 50 μ L.

- g. 3–5 μL of 100 U/mL DNase I (need to establish amount for each new batch).
- h. 1 μL of 10 U/ μL DNA Polymerase I.
2. Mix well.
3. Incubate at 15°C for 90 min.
4. Stop reaction by placing tubes on ice.
5. Check the size of the labeled products by running an aliquot on a 2% agarose gel (in TBE and containing 5 μL of 5 mg/mL ethidium bromide/100 mL) as follows:
 - a. 5 μL of labeled probe (approx 100 ng).
 - b. 4 μL of gel-loading buffer (5X bromophenol blue).
 - c. 11 μL of sterile distilled water.
6. Run at 50 V for 1–1.5 h with *PhiX174 HaeIII* (20 μL = 250 ng) as a size marker.
7. View on a transilluminator and photograph. The optimal size range for *in situ* hybridization is 50–500 bp (see **Note 4**). A smear of products from 100 to 300 (corresponding to the six smallest bands of *PhiX174*) is suitable. If the size range is larger than this, add a further 5 μL of DNase I, place at 15°C for an additional 30–60 min, and run another aliquot on a gel to test the size.
8. Purify to remove unincorporated nucleotides by passing the labeled probe through a MicroSpin G50 column (designed for biotinylated probes) according to the manufacturer's instructions.
9. Measure the volume of eluate and then ethanol precipitate the purified, labeled probe by adding the following:
 - a. 50 μg of *E. coli* tRNA.
 - b. 50 μg of salmon sperm DNA.
 - c. 0.1 vol of 3 M sodium acetate, pH 5.6.
 - d. 2–2.25 vol of ice-cold ethanol.Mix well and place at –70°C for 1–2 h or –20°C overnight.
10. Centrifuge in a microcentrifuge for 15–25 min at 4°C. Pour off the supernatant and dry the pellet (either air-dry or dry in a vacuum desiccator). Resuspend the pellet in 20 μL TE pH 8.0 to give a final concentration of 50 ng/ μL . Allow the DNA to dissolve at room temperature for 1 to 2 h or at 4°C overnight with occasional mixing. Purified, labeled probes are stable for several years when stored at –20°C.

3.5. Competitive In Situ Suppression Hybridization (see Note 5)

Clones containing large DNA fragments (i.e., phage, cosmid, YAC, P1) and whole-chromosome paints require an additional step before hybridization to remove ubiquitous repetitive sequences (see **Table 1**) This is achieved by a short incubation prior to hybridization, with unlabeled human competitor DNA, in the form of either total human DNA (placental DNA, sheared and sonicated to 50–300 bp) or human Cot-1 DNA (Gibco-BRL). When all of the probe sequences contribute to the hybridization signal (e.g., repetitive DNA probes, unique cDNA probes), there is no need to add competitor DNA. Suggested amounts of probe and competitor DNA are given in **Table 1**. Hybridization is carried out in a moist chamber. This can be achieved by using a plastic micro-

Table 1
Suggested Amount of Probe and Competitor (10 μ L Hybridization Volume)

Type of probe	Amount	Cot-1 DNA (μ g)
Single fragment cloned in plasmid ^a	200 ng	
Single fragment cloned in phage	200 ng	2.5
Single fragment cloned in cosmid	50–100 ng	2.5
YAC, total yeast DNA	400 ng–1 μ g	5–7.5
PAC, P1	200–400 ng	3–5
Alphoid DNA repeat (centromere) ^a	10 ng	
Whole-chromosome paint, libraries ^b	100–400 ng	5–7.5
Whole-chromosome paint, PCR amplified flow-sorted chromosomes	100 ng	6.25

^aWhole probe contributes to signal; no competitor required.

^bDNA from flow-sorted chromosome libraries cloned in plasmids.

scope slide box containing moist tissue paper (wring out excess water), placed in an incubator or floated in a water bath. Alternatively, we use metal trays (Lamb's immunoslide staining trays, Raymond Lamb, UK) for both hybridization and detection steps.

1. Dry down the appropriate concentration of probe and competitor either in a vacuum desiccator (Speedivac) or by ethanol precipitation; e.g., for cosmids:
 - a. 100 ng of labeled probe.
 - b. 2.5 μ g (2.5 μ L) of Cot-1 DNA.
 - c. 0.1 vol of 3 M sodium acetate.
 - d. 2 vol of ice-cold ethanol.
 Allow to precipitate for 1 to 2 h at -70°C .
2. Centrifuge and dry down the pellet as for labeled probes. Resuspend the pellet in 11 μ L of hybridization buffer (warmed to room temperature).
3. Denature the probe mixture at 95°C in a hotblock for 10 min. Plunge the tubes on ice for a few minutes, and then centrifuge briefly in a microcentrifuge.
4. Place the probe mixture at 37°C for 15 min to 2 h.
5. Just prior to hybridization, denature the chromosomal DNA as follows:
 - a. Incubate the slides in denaturing solution (in a water bath in a fume hood) at 70°C for 5 min.
 - b. Wash the slides in cold 2X SSC, followed by two changes of 2X SSC.
 - c. Dehydrate through a cold alcohol series (70%, 90%, absolute).
6. Air-dry the slides and place on a hot plate at approx 42°C .
7. Centrifuge the probe mixture quickly to get the liquid to the bottom of the tube. Place this mixture on the previously treated slide containing chromosomes and cover with a 22×32 mm coverslip (do not let drop dry). Seal the coverslip with rubber solution, and place the slides in a moist chamber at 37°C for overnight to 4 d.

8. Remove the rubber solution. The coverslips can then be removed either by soaking in 2X SSC or by gently tipping them off into the glass disposal bin (never pull them off).
9. Carry out the following washes (*see Note 6*):
 - a. Three washes (3 min each) in 2X SSC at room temperature (with agitation).
 - b. Two washes (20 min each) in 0.1X SSC at 65°C.
 - c. One 5-min wash in 0.1X SSC at room temperature (with agitation).
10. Wash the slides in wash solution for 3 min.
11. Incubate the slides in blocking solution for 10–20 min (room temperature).
12. Wash in wash solution for 3 min before carrying out the appropriate detection steps.

3.6. Detection of Bound, Labeled Probe

For directly fluorochrome labeled probes, no immunologic detection steps are required. For repetitive centromeric probes and whole-chromosome paints, usually only one layer of detection reagent is required (i.e., fluorochrome-conjugated avidin or antibody). For single-copy probes, we use the following protocols, using three detection layers. The signal can be amplified further by adding several layers of detection reagents. However, increasing the number of layers to more than three will result in high background and reduced signal:noise ratio.

3.6.1. Biotin-Labeled Probes

1. Dilute 2.5 μL of stock avidin DCS-FITC in 1 mL of blocking solution (final concentration: 5 $\mu\text{g/mL}$). Add 100 μL of this under a 24 \times 50 mm coverslip. Incubate in a moist chamber at 37°C for 30 min.
2. Flick off the coverslips and wash the slides three times (for 3 min each) in wash solution (*see Subheading 2.5.*).
3. Dilute 10 μL of stock biotin anti-avidin D in 1 mL of blocking solution (final concentration: 5 $\mu\text{g/mL}$). Add 100 μL of this under a 24 \times 50 mm coverslip. Incubate in a moist chamber at 37°C for 30 min.
4. Flick off the coverslips and wash the slides three times (for 3 min each) in wash solution.
5. Add 100 μL of avidin-FITC (same as the first layer). Incubate for 30 min as before.
6. Carry out the following final washes:
 - a. Wash once for 3 min in wash solution.
 - b. Wash twice (5 min each) in PBS.
 - c. Dehydrate the slides through an ethanol series. Air-dry.
7. Mount the slides in 40 μL of Vectashield containing 1.5 $\mu\text{g/mL}$ of DAPI and 0.75 $\mu\text{g/mL}$ of propidium iodide under a 24 \times 50 mm coverslip. Seal the edges of the coverslip with rubber solution or nail varnish. The signal keeps well for several weeks when slides are stored at 4°C.

3.6.2. Digoxigenin-Labeled Probes

1. Prepare all antibody dilutions in blocking solution, filtered before use. Make up the following antibody dilutions in 1 mL of blocking solution:
 - a. First layer: 1.5 μ L of mouse monoclonal antidigoxigenin.
 - b. Second layer: 1 μ L of rabbit antimouse-FITC.
 - c. Third layer: 10 μ L of monoclonal antirabbit-FITC.
2. Incubate in each antibody layer (100 μ L under a 24 \times 50 mm coverslip) for 30 min at 37°C in a moist chamber.
3. After each antibody layer, wash three times (3 min each) in wash solution.
4. Carry out the final washes as for biotin detection
5. Mount in Vectashield containing 1.5 μ g/mL of DAPI and 0.75 μ g/mL of propidium iodide.

3.6.3. Dual-Color Detection of Biotin- and Digoxigenin-Labeled Probes

1. Prepare all antibody dilutions in blocking solution, filtered before use. Make up the following antibody dilutions in 1 mL of blocking solution:
 - a. First layer: 1 μ L of avidin-Texas red + 1.5 μ L of mouse monoclonal antidigoxigenin.
 - b. Second layer: 10 μ L of biotin antiavidin + 1 μ L of rabbit antimouse-FITC.
 - c. Third layer: 1 μ L of avidin-Texas red + 10 μ L of monoclonal antirabbit-FITC.
2. Incubate in each antibody layer for 30 min at 37°C in a moist chamber.
3. After each antibody layer, wash three times (3 min each) in wash solution.
4. Carry out the final washes as for biotin detection.
5. Mount in Vectashield containing only 1.5 μ g/mL of DAPI.

3.7. Microscopy

For the majority of FISH signals, the only equipment required is an epifluorescence microscope equipped with the appropriate filter sets (*see Table 2* for commonly used fluorochromes and their spectral characteristics). Both metaphase and interphase FISH analysis can be performed directly at the microscope, with photographic recording of representative images. However, photomicroscopy of multicolor FISH images may be difficult, owing to the long exposure times and loss of registration of images when changing filters. Digital imaging fluorescence systems such as confocal laser scanning microscopes and charge-coupled device (CCD) cameras provide significant advantages in terms of both image storage and the ability for image processing. Confocal laser scanning microscopes provide complete and accurate registration of fluorescent signals on chromosomes by the simultaneous scanning of each fluorochrome through separate filter blocks. These systems are also highly suitable for three-dimensional FISH applications. However, confocal systems are limited for multicolor imaging because most standard lasers only allow excitation of up to three fluorochromes.

Table 2
Fluorescent Dyes Commonly Used for FISH

Fluorochrome	Color	Absorbance (nm)	Emission (nm)
DAPI	Blue	350	456
SpectrumAqua ^a	Blue	433	480
FITC	Yellow/green	490	520
SpectrumGreen ^a	Green	497	524
Rhodamine	Red	550	575
Cy3 ^b	Red	554	568
SpectrumOrange ^a	Orange	559	588
Cy3.5 ^b	Red	581	588
SpectrumRed ^a	Red	587	612
Texas red	Deep red	595	615
Cy5 ^b	Far red	652	672
Cy5.5 ^b	Near infrared	682	703
Cy7 ^b	Near infrared	755	778

^aVysis.

^bAmersham.

High-performance, highly cooled (-30°C) CCD cameras are extremely sensitive to photons over a wide range of wavelengths and are now the instrument of choice for FISH, particularly for multicolor applications. Problems with image registration owing to the movement of microscope filter blocks can be overcome by the use of a filter wheel containing the excitation filters and situated between the lamp and the microscope. For most FISH applications ambient temperature ($+15^{\circ}\text{C}$), video-rated CCD cameras are probably sufficient, and for whole-chromosome painting, relatively inexpensive video cameras will suffice. When purchasing a FISH imaging system, it is important to consider requirements for hardware (i.e., compatibility and storage) and software (i.e., sophisticated packages for multicolor FISH and comparative genomic hybridization in addition to standard image capture and enhancement facilities).

3.8. Interpretation of Results

3.8.1. Metaphase FISH

For mapping purposes and also for the assessment of yeast artificial chromosome (YAC) chimerism, FISH is carried out to normal male metaphase spreads. To determine the number of metaphases that need to be evaluated for these applications, it is important to consider the hybridization efficiency, which decreases proportionately with probe size. For whole-chromosome painting probes and centromeric alphoid repeats, only a few metaphases need to be evaluated. Single-copy probes cloned in cosmids, YACs, bacterial artifi-

cial chromosomes (BACs), P1, and PACs also hybridize very efficiently (>80% of cells with signal on all four chromatids), so that usually only a few cells (5–10) need to be scored. Small single-copy sequences (<3 kb) hybridize less efficiently (30% of cells with signal on all four chromatids), and, thus, many more metaphases need to be evaluated.

In addition to these considerations, it should be borne in mind that all leukemic cell preparations (with the possible exception of cell lines) are heterogeneous mixtures, with variable numbers of normal and clonal cells. Therefore, for assessing the presence of numerical or structural rearrangements in leukemic bone marrow, it is important to screen as many metaphases as possible. The percentage of abnormal cells from G-banding can be used as a guide. For the presence of deletions, the normal chromosome homolog serves as an internal control. For mapping the extent of chromosome deletions, it is necessary to include a probe to tag the appropriate chromosome, so that only metaphases with the abnormal chromosome are scored (*see Fig. 1B*).

3.8.2. Interphase FISH

Probes used for interphase analysis should be chosen to hybridize with high efficiency (>90%). Also note that in dual- or triple-color FISH experiments, three probes with 90% efficiency will hybridize simultaneously to only 73% of nuclei. Centromeric probes are most suitable for detecting numerical abnormalities in interphase, because these exhibit compact, unambiguous signals. Choosing suitable probes is particularly important for the assessment of deletions. In this case, cohybridization with a control probe in a second color will increase the sensitivity. The control probe should be of similar complexity, localized to a region not likely to be affected by a chromosome rearrangement in the particular type of leukemia being studied. However, because of the established occurrence of false monosomy (owing to inefficient hybridization or the overlap of signals viewed in two dimensions), diagnostic cutoff levels need to be established for each probe. DNA probes are now available commercially for the majority of specific translocations in leukemia. Differential labeling and dual-color detection of these allow the direct visualization of the fusion gene. However, it is important to establish “in-house” cutoff levels for false positivity for such probes. This can be quite high for some translocations, owing to the variability of breakpoints.

3.9. Advanced Methods and Applications

3.9.1. Whole-Chromosome Painting Probes by Degenerate Oligonucleotide Primer-PCR Amplification of Flow-Sorted Chromosomes (8)

One of the most significant advances in probe generation has been the ability to selectively amplify genomic regions by PCR. Whole-chromosome paints

can be produced by interspersed repetitive sequence element-PCR (e.g., Alu-PCR) by selectively amplifying the human DNA content of somatic cell hybrids (21). However, because of the distribution of these sequences across the genome, the resultant chromosome paints produce an R-banded pattern, which may not be optimal for the detection of some rearrangements. The technique of degenerate oligonucleotide primer (DOP)-PCR can be used to obtain more evenly distributed whole-chromosome or region-specific chromosome paints (8,9). This technique is also the basis for the production of 24 color paint sets for SKY (see Chapter 3) and M-FISH.

3.9.1.1. FIRST-ROUND DOP-PCR AMPLIFICATION

All reagents except chromosomal DNA and *Taq* 1 polymerase can be sterilized by exposure to short-wave ultraviolet irradiation (5 min on a transilluminator). All solutions, microcentrifuge tubes, and tips should be autoclaved and kept for only PCR. Use aerosol-resistant tips and add all reagents in a laminar flow hood to minimize contamination. Prepare positive (2.5 pg of genomic DNA) and negative (all of the reagents except chromosomes) controls in the same way.

1. Combine in a sterile 0.5-mL microcentrifuge tube: x μ L (= 500 flow-sorted chromosomes), 50 μ L of 2X PCR buffer, 10 μ L of dNTP mix, 6.6 μ L of 30 mM 6-MW primer, 0.5 μ L of (1.25 U) *Taq* 1 polymerase, and distilled water to a final volume 100 μ L.
2. Overlay with 100 μ L of mineral oil and run the following program in a DNA thermal cycler: Denature for 10 min at 93°C. 5 cycles of: 1 min at 94°C, 1.5 min at 30°C, 3 min at 30–72°C transition, and 3 min at 72°C; 35 cycles of 1 min at 94°C, 1 min at 62°C, and 3 min at 72°C, with an additional 1 s/cycle and final extension time of 10 min.
3. Run a 10- μ L aliquot of the amplified products on a 1.2% agarose gel with *Phi*X174 to check the success of the amplification. There should be no amplification in the negative control.

3.9.1.2. SECOND-ROUND DOP-PCR AND PROBE LABELING

1. Add the following to a new sterile 0.5-mL microcentrifuge tube: 5 μ L of amplified products from first round, 25 μ L of 2X PCR buffer, 5 μ L of nucleotide mix, 3.3 μ L of 6-MW primer, 2.5 μ L *Taq* 1 polymerase, and 12 μ L of 1 mM biotin-16-dUTP.
2. Mix well, overlay with 50 μ L of mineral oil, and run the following PCR program: Denature for 10 min at 93°C. 25 cycles of 1 min at 94°C, 1 min at 62°C, and 3 min at 72°C, with a final extension time of 10 min.
3. Remove the mineral oil. Run 10 μ L of labeled products on a 1.2% agarose gel to check the size range. If the labeled fragments are too large, recut with 5 μ L of DNase I for 30–60 min.
4. Purify the labeled DNA through a MicroSpin G50 (or Sephadex G50) column. Measure the DNA concentration of the purified, labeled DNA in a fluorimeter

(usually 20–50 ng/mL). Ethanol precipitate the labeled DNA with tRNA and single-stranded DNA as usual, and dry and resuspend in distilled water or TE to a suitable concentration; this is now ready for use as a chromosome paint. Use 100 ng of probe + 6 µg of Cot-1 DNA per slide.

3.9.2. Combined Immunophenotyping and FISH

The following technique relies on the ability of the reaction product of the APAAP immunophenotyping method to remain throughout subsequent harsh FISH procedures (22). Staining with Fast red produces autofluorescence visible through all filter sets and can be viewed at the same time as the FISH signal. We have used this to identify the cell lineages carrying the del(5q) clonal chromosome abnormality in myelodysplastic syndrome patients (*see Fig. 1C,D*). After immunostaining, FISH is carried out essentially as described in **Subheadings 3.5. and 3.6.**, using a pepsin pretreatment to aid probe penetration.

1. Allow bone marrow smears to reach room temperature and then unwrap.
2. Fix in either acetone:methanol (1:1) for 90 s or acetone alone for 10 min. Then transfer immediately to TBS for 5 min at room temperature.
3. Add the appropriate primary mouse monoclonal antibody and incubate the slides in a moist chamber at room temperature for 30 min. Also incubate a negative control slide (no antibody added) in PBS for 30 min.
4. Add the second layer, rabbit antimouse antibody, and incubate for 30 min in a humid chamber.
5. Add the third antibody, mouse monoclonal APAAP complex, and incubate for 30 min in a moist chamber at room temperature.
6. Wash for 5 min in TBS between each antibody layer.
7. To enhance staining, repeat the antimouse antibody and APAAP steps (**steps 4 and 5**) with reduced incubation times of 10 min.
8. Finally, add alkaline phosphatase substrate to the slides and incubate for 10–20 min.
9. Wash the slides in TBS, then distilled water, and allow to air-dry.

3.9.3. Multicolor FISH

The simplest approach to multicolor FISH uses two probes labeled with different haptens or fluorochromes, and the third probe labeled separately with both, mixed in a 1:1 ratio. An extension of this approach can be used to detect up to seven different targets using three fluorochromes (23). Increasing the number of fluorochromes to five allows the identification of all 24 pairs of human chromosomes (16,17). Both M-FISH and SKY use a set of whole-chromosome paints combinatorially labeled with five fluorochromes, but differ in their method for the discrimination of the fluorochrome combinations. The SKY approach is detailed in Chapter 3. The second detection method, M-FISH, uses a filter-based detection system, capturing the separate fluorochrome images for each of five fluorochromes using specifically selected narrow

bandpass filter sets. We have used M-FISH and a set of combinatorially labeled whole-chromosome paints to analyze the complex karyotype in the myeloid leukemia-derived cell line GF-D8 (24) (see Fig. 1E).

4. Notes

1. Proper storage of slides is important to maintain good-quality chromosomal DNA. Slides can be used for hybridization the day after they are made, or kept for up to 1 mo at room temperature. For long-term storage, keep slides in a sealed container with desiccant at -20°C .
2. Alu-PCR amplification of total yeast DNA can be used to increase the yield of YAC DNA (25). However, Alu-poor YACs may not amplify, and the sensitivity for determining YAC chimerism is not known. Isolation of the YAC from the yeast background by pulsed-field gel electrophoresis can be used, but this has a low yield and may be difficult if the YAC is not visible by ethidium bromide staining.
3. It is important to have an accurate measurement of DNA concentration for nick translation labeling. Spectrophotometric measurements are often inaccurate, owing to RNA and other contaminants. We measure probe DNA concentration using a fluorometer (e.g., Hoeffer DynaQuant 200; Amersham Pharmacia Biotech), which measures the fluorescence of a DNA-binding dye, compared to a known standard.
4. These are frequently encountered problems:
 - a. *No hybridization signal.* This may be owing to insufficient probe DNA in the hybridization mix. The DNA concentration of any new probe should be measured accurately. This may also be owing to inadequate denaturation of probe and/or chromosomes.
 - b. *Probe fragment size too small.* Always check the labeled fragment size on a 2% (1.2% for PCR products) gel with *PhiX174 HaeIII* size marker. The optimum fragment size is 100–500 bp.
 - c. *High background.* High background with strong specific signal may be owing to low stringency of hybridization or posthybridization washes or incomplete competition. The stringency of hybridization can be increased by either increasing the hybridization temperature, increasing the formamide concentration of the hybridization mix and/or posthybridization washes to 60%, or decreasing the SSC concentration to 0.1% in the posthybridization washes. Alternatively, increase the Cot-1 DNA concentration: This is already present in large excess so that any increase should be substantial (up to 10-fold).
 - d. *Brightly fluorescent signal all over the slide.* This occurs when the labeled probe fragments are too large: If labeled probe is >500 bp, it should be recut with DNase I. High background of this type also may be caused by insufficient blocking with BSA.
 - e. *Cells lost from slide.* Handle slides with care at all stages especially during removal of cover slips (never pull them off). Agitation during posthybridization washes should be carried out on a rocking platform set at minimum speed.

- f. *Poor chromosome morphology/banding.* If chromosomes look “blown,” they may have been overdenatured: Always check the temperature of the denaturing solution inside the coplin jar. Overdenatured chromosomes give a C-banding pattern with DAPI staining.
5. These are important hybridization parameters:
 - a. *Temperature.* The temperature at which two DNA strands separate (T_m) is in the range of 85–95°C. The optimal DNA-DNA reassociation temperature (T_r) is approx 25°C below the T_m of the native duplex. However, fixed chromosome preparations on microscope slides will not tolerate temperatures >65°C for long periods. The presence of formamide in the hybridization buffer lowers the T_r , allowing hybridization to take place at 37–42°C, and preserving chromosome morphology.
 - b. *Time of hybridization.* This depends on the size and copy number of the target sequence, as well as the complexity of the probe. Repetitive sequence probes such as alphoid centromeric probes require only 1 h for hybridization. Unique sequence probes cloned in plasmid, cosmid, or phage vector require hybridization overnight (16–18 h). Larger insert probes (large YACs) may benefit from longer times to (1–2 d), and very complex probes such as multicolor painting sets and whole genomes in comparative genomic hybridization require 2 to 3 d.
 - c. *Denaturation of chromosomal DNA.* The optimal time for denaturation needs to be determined for each batch of slides. Overdenaturation results in loss of chromosome morphology and very poor DAPI banding after hybridization. Underdenaturation results in little or no signal. The pH of the denaturation solution is also important: This should be checked when the solution is up to temperature and adjusted if necessary. It is preferable to prepare the denaturing solution and use it as soon as it has reached the desired temperature to prevent pH fluctuations. Alternatively, use EDTA (final concentration of 0.1 mM) to stabilize the denaturing solution against pH changes.
 - d. *Stringency of hybridization conditions.* Renaturation depends on specific base pairing between two complementary DNA strands and can be controlled by the stringency of the hybridization conditions. Increasing the hybridization temperature or decreasing the salt concentration increases the stringency, which has a direct effect on the accuracy of base pairing.
6. The low salt washes used in **Subheading 3.5.** are comparable to the following formamide washes: 50% formamide in 2X SSC (three times for 5 min each), followed by 2X SSC (three times for 5 min each) at 45°C.

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