Detection of Genetic Alterations by ImmunoFISH Analysis of Whole Cells Extracted from Routine Biopsy Material

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The detection of genetic abnormalities (eg, translocations, amplifications) in paraffin-embedded samples by the fluorescence *in situ* hybridization (FISH) technique is usually performed on tissue sections. FISH analysis of nuclei extracted from paraffin-embedded samples is also possible, but the technique is not widely used, principally because of the extra labor involved and the loss of information on tissue architecture. In this article, we report that nuclei extracted from paraffin-embedded tissue often retain at least part of the surrounding cytoplasm. Consequently, immunocytochemical labeling for a range of cellular markers (eg, of lineage or proliferation) can be performed in combination with FISH labeling, allowing specific cell populations to be analyzed for genetic abnormalities. These cell preparations are largely free of the problems associated with tissue sections (eg, truncation artifact, signals in different focal planes) so that interpretation is easy and numerical chromosomal abnormalities are readily assessed. Cells isolated from paraffin sections can be stored in suspension so that arrays can be created as and when needed from a range of neoplasms for investigation by the immunoFISH technique (for example, for studying a new genetic abnormality). This procedure represents a novel methodology, which in some settings offers clear advantages over analysis of tissue sections. (*J Mol Diagn* 2007, 9:479–489; DOI: 10.2353/jmoldx.2007.070041)

The fluorescence *in situ* hybridization (FISH) technique is now widely used in clinical practice to detect amplification of the *ERB2* gene in paraffin-embedded tissue sections of breast carcinoma, but it also finds many other applications, including the detection of chromosomal translocations in lymphomas and soft tissue tumors.^{1–5} However, a number of cells inevitably lose part of their nuclear material during tissue sectioning, resulting in incomplete FISH labeling patterns.^{6,7} In addition the optimal conditions for proteolytic digestion (used to reduce nonspecific labeling and to improve labeling intensity) often vary from one biopsy to another and even within a single section, so that problems of interpretation caused by under- or overdigestion are not uncommon.

Even when *in situ* hybridization is technically satisfactory, the interpretation of results may be complicated not only by nuclear truncation artifacts but also by difficulties in distinguishing closely packed and overlapping nuclei and in assessing signals in different focal planes.^{8–13} For these reasons, some laboratories perform FISH analysis on nuclei isolated from tissue blocks (after dewaxing and proteolytic digestion).^{14–28} However, the extra technical work involved and the loss of tissue architecture means that the use of tissue sections is generally preferred.^{1,4}

In the present article, we report that proteolytic digestion is not mandatory when extracting nuclei from dewaxed tissue, thus reducing the labor involved. In addition, we note that the extraction technique frequently yields many cells that are largely intact, with the consequence that the identity of cells bearing different FISH labeling patterns can be ascertained by

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performing immunofluorescence labeling before hybridization with probes. The assessment of numerical abnormalities is easier than in tissue sections; furthermore, isolated cells can be stored in suspension without loss of antigenicity or reactivity by the FISH technique. Thus, in a research setting, arrays of isolated cells can be produced when required (eg, for screening for a newly identified genetic abnormality in multiple samples). For these reasons, we suggest that cells isolated from routine biopsies may offer advantages for FISH studies that have been overlooked, both in a routine and research setting.

Materials and Methods

Tissues

Formalin-fixed, paraffin-embedded tissue samples of tonsil ($n = 3$), follicular lymphoma ($n = 5$), splenic marginal zone lymphoma ($n = 2$), diffuse large B-cell lymphoma ($n = 6$), mantle cell lymphoma ($n = 3$), Burkitt's lymphoma ($n = 2$), Hodgkin's lymphoma ($n = 2$), and lymph node ($n = 3$) were obtained from the archives of the Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, and from Professor M.L. Hansmann (Institute of Pathology, University Clinic, Frankfurt am Main, Germany), Professor J.H. van Krieken (Department of Pathology, University Medical Centre, Nijmegen, The Netherlands), and Professor S. Pileri (Unit of Hematopathology, University of Bologna, Bologna, Italy). A bone marrow trephine from a case of chronic myeloid leukemia was obtained from the Hematology Department, Addenbrooke's Hospital, Cambridge, UK.

Antibodies

The primary antibodies used in this study, along with the dilutions and sources, are listed in Table 1.

FISH Probes

Fluorescence *in situ* hybridization and immunoFISH experiments were conducted using split-apart probes (Dako A/S, Glostrup, Denmark) for the following genes: *BCL2*, *BCL6*, *IGH*, *C-MYC*, *CCND1*, *MALT1*, and *PAX5*. In addition, dual-color, dual-fusion probes for the *IGH-CCND1* and *BCR-ABL* fusion genes (Vysis, Abbott Molecular, Maidenhead, UK) and centromeric probes against chromosomes 3, 8, and 11 (Vysis) were used.

Isolation of Cells from Paraffin-Embedded Biopsies

A tissue microarray needle (Beecher Instruments Inc., Sun Prairie, WI) (1 mm in diameter) was used to remove cores (one or two per sample) from paraffin-embedded tissue blocks. In one experiment, 10- μ m-thick sections from bone marrow were used, and they were handled as if they were tissue cores. Cores were placed in a 1.5-ml Eppendorf tube before dewaxing and further processing as previously de-

scribed.²¹ Paraffin wax was removed by three 10-minute incubations in xylene (Genta Medical, York, UK) or CitrocLEAR (HD Supplies, Aylesbury, UK), and the tissue was hydrated in 95, 75, and 50% ethanol (3 minutes for each step). The sample was manually homogenized in a small volume of 50% ethanol for 2 minutes by rotation up and down in the tube using the end of a plastic universal spatula (Alpha Laboratories Ltd., Eastleigh, UK). In some experiments, samples were digested using proteinase K (S3020; Dako A/S) for up to 30 minutes at 37°C, being vortexed at intervals for 5 seconds. Phosphate-buffered saline (PBS; Oxoid, Basingstoke, UK) was added to each tube before centrifugation at 6000 rpm for 10 minutes. The supernatant was removed and the pellet resuspended in PBS. This material was then passed through a 30- or 50- μ m Filcon nylon mesh (BD Biosciences, Oxford, UK) and centrifuged again (as described above) before removing the supernatant. Methanol/acetic acid (3:1 ratio, 100 to 300 μ l) was added to each sample before storage at -70°C . To avoid clumping of nuclei when placed on slides, the methanol/acetic acid was replaced with PBS after removal from storage.

Transfer of Cell Suspensions to Microscope Slides

Small circular rings (diameter approximately 0.2 to 1.0 cm) were created on X-tra adhesive microscopic slides (Surgipath, Peterborough, UK) with a PAP pen (BioGenex, San Ramon, CA). Aliquots (3 to 5 μ l) from nuclear suspensions were mixed with 4 μ l of 10% poly-L-lysine (Sigma-Aldrich, St. Louis, MO) before being placed into the rings and dried at room temperature ("spotting method"). The slides were then heated at 60°C for 30 minutes, cooled, and wrapped in foil for storage at -20°C . In a few experiments, the isolated nuclei were deposited on slides by cytocentrifugation in a Shandon Cytospin 2 (5 minutes at 550 rpm; Thermo Fisher Scientific Inc., Waltham, MA).

Immunostaining of Isolated Cells

Immunoperoxidase Technique

Slides were pretreated with a conventional antigen retrieval protocol by incubation in 0.01 mol/L citrate buffer (pH 6.0) or Tris/ethylenediamine tetraacetic acid buffer (pH 9.0) in either a microwave oven (20 minutes, 750 W) or a pressure cooker (2 minutes). They were then immunostained by a conventional immunoperoxidase procedure using a panel of primary antibodies (Table 1) and horseradish peroxidase-conjugated secondary anti-Ig antibodies (Dako A/S). In negative controls, the primary antibody was omitted. Cryostat sections of tonsil were used as a positive control for the reactivity of primary antibodies.

Double Immunofluorescence Technique

Slides were pretreated as described above and then incubated for 45 minutes at room temperature with pairs

Table 1. List of Primary Antibodies Used for Flow Cytometry, Immunohistochemistry, Double Immunofluorescence, and ImmunofISH

Antibody	Clone, catalog no.	Dilution	Source	Immunostaining pattern
Bcl2 (mouse monoclonal, IgG1)	124	1:100	Author's laboratory (DYM)	Cytoplasmic
BOB1 (rabbit polyclonal)	C-20, sc-955	1:1000	Santa Cruz Biotechnology, Santa Cruz, CA	Nuclear
CD3 (rabbit polyclonal)	A0452	1:100	Dako A/S	Surface membrane*
CD5 (mouse monoclonal, IgG1)	CD5/54/F6	Undiluted	Author's laboratory (DYM)	Surface membrane*
CD5 (rabbit monoclonal)	SP19, RM-9119-S	1:100	Lab Vision, Fremont, CA	Surface membrane*
CD15 (mouse monoclonal, IgM)	By87a	Undiluted (supernatant)	Author's laboratory (DYM)	Surface membrane*
CD20 (mouse monoclonal, IgG2a)	L26, M0755	Undiluted (supernatant)	Dako A/S	Surface membrane*
CD30 (mouse monoclonal, IgG1)	Ber-H2, M0751	Undiluted	Dako A/S	Surface membrane*
CD30 (mouse monoclonal, IgG2a)	CON6D/B5	1:100	G. Roncador (National Cancer Research Centre, Madrid, Spain)	Surface membrane*
CD34 (mouse monoclonal, IgG1)	QBEnd-10, M7165	1:100	Dako A/S	Surface membrane*
CD79a (mouse monoclonal, IgG1)	JCB117, M7050	Undiluted	Dako A/S	Surface membrane*
CD235a/glycophorin A (mouse monoclonal, IgG1)	JC159, M0819	1:100	Dako A/S	Surface membrane*
CD236R/glycophorin C (mouse monoclonal, IgG1)	Ret40f, M0820	1:100	Dako A/S	Surface membrane*
CCND1/cyclin D1 (rabbit monoclonal)	SP4, RM-9104-S	1:100	Lab Vision	Nuclear
Ki-67 (rabbit polyclonal)	ab833	1:100	Abcam, Cambridge, MA	Nuclear
Ki-67 (mouse monoclonal, IgG1)	MIB-1, M7240	1:100	Dako A/S	Nuclear
LAT/linker for activation of T cells (mouse monoclonal, IgG1)	LAT-01	1:50	G. Delsol (INSERM U563, Toulouse, France)	Cytoplasmic
Myeloperoxidase (rabbit polyclonal)	A0398	1:200	Dako A/S	Cytoplasmic
Oct2 (rat monoclonal)	SA223	1:10	L. Corcoran (Walter and Eliza Hall Institute, Melbourne, VIC, Australia)	Nuclear
Pax-5 (mouse monoclonal, IgG1)	24, 610863	1:50	BD Biosciences, San Jose, CA	Nuclear
p63/VS38c (mouse monoclonal, IgG1)	VS38c, M7077	1:100	Dako A/S	Cytoplasmic
SLP76 (rabbit polyclonal)	H-300, sc-9062	1:500	Santa Cruz Biotechnology	Cytoplasmic
von Willebrand factor (rabbit polyclonal)	A0082	1:50	Dako A/S	Cytoplasmic

*Some surface membrane molecules may also be present in an intracellular location and may therefore be detectable in isolated cells even when the surface has been lost.

of primary antibodies that were either from different species (eg, mouse and rabbit) or of differing immunoglobulin isotypes/subclasses (eg, IgG1, IgG2a, IgM), as previously described.²⁹ Slides were then washed in PBS for up to 5 minutes and incubated in the dark for 45 minutes with pairs of species-, isotype-, or subclass-specific secondary antibodies, conjugated to a green (Alexa Fluor 488) or red (Alexa Fluor 568) fluorochrome (Molecular Probes, Leiden, The Netherlands). The slides were washed in PBS for up to 5 minutes, mounted in fluorescence mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA) and examined on an E800 Eclipse

fluorescence microscope (Nikon, Kingston-on-Thames, UK) equipped with Nikon filter blocks for blue, green, and red fluorescence (blocks UV-2E/C, B-2E/C, and Y-2E/C, respectively). Images were captured with an AxioCam charge-coupled device camera and Axiovision software (Imaging Associates, Bicester, UK) and then processed using the Photoshop program (Adobe, San Jose, CA).

Flow Cytometry

Cell samples were pretreated in a pressure cooker (see above) and then suspended in primary antibodies for incubation overnight at 4°C. Samples were then

washed twice in PBS, resuspended in a fluorescently labeled secondary antibody (Alex Fluor 488; Molecular Probes) and incubated in the dark for one hour at 4°C. After two washes in PBS, samples were resuspended in PBS containing 0.1% bovine serum albumin (Sigma-Aldrich) and analyzed on a Becton Dickinson FACSCalibur (Becton Dickinson UK Ltd., Oxford, UK) flow cytometer.

Electron Microscopy

Suspensions of isolated nuclei were prepared as described above and then fixed overnight in 4% (v/v) glutaraldehyde (Agar Scientific, Stansted, UK) in 0.1 mol/L phosphate buffer. After washing in 0.1 mol/L phosphate buffer, the nuclei were postfixed for 1 hour in 1% (w/v) osmium tetroxide dissolved in phosphate buffer, dehydrated in a graded ethanol series, treated with propylene oxide, and embedded in Spurr's epoxy resin (Agar Scientific). Thin sections were cut, contrasted with uranyl acetate and lead citrate, and examined in a JEOL 1200EX transmission electron microscope (JEOL UK Ltd., Welwyn Garden City, UK) at 75 kV.

FISH Procedures

Fluorescence *in situ* hybridization was performed using the reagents in the Dako FISH ancillary kit (Dako A/S) according to the manufacturer's instructions. Slides were mounted using Vectashield fluorescence mounting medium containing DAPI (Vector Laboratories Inc.) and viewed using a fluorescence microscope equipped with a dual pass fluorescein isothiocyanate/rhodamine filter and a UV longpass filter (Chroma Technology Corp, Rockingham, VT).

ImmunoFISH Procedure

All incubations were performed at room temperature in the dark and interspersed with 5-minute washes in PBS. Slides were first incubated for 30 minutes with the primary antibody. Depending on the species and type of the primary antibody, they were then incubated for 45 minutes with biotin-conjugated goat anti-mouse/rabbit Ig (Biotinylated Link, Dako A/S K1015), or biotinylated rabbit anti-rat immunoglobulin (1:100 dilution, Dako A/S E0467) followed by a mixture of Alexa Fluor 350-conjugated streptavidin and Alexa Fluor 350-conjugated goat anti-mouse/rabbit/rat immunoglobulins (Molecular Probes), both diluted 1:100 for 45 minutes. After another wash, the slides were incubated with Alexa Fluor 350-conjugated donkey anti-goat immunoglobulin for another 45 minutes. The slides were washed and then subjected to dehydration and hybridization as described above. Following a stringency wash the following day, the slides were mounted using Vectashield mounting medium for fluorescence (without DAPI) (Vector Laboratories, Inc.) and then examined and photographed as described above. The positions of antigen-negative cells were identified because of their near-background signals in one of the

fluorescence channels, and the corresponding outlines were pasted into the final three color images.

Results

Isolation of Cells from Paraffin Sections

We were able to extract nuclei from all lymphoid tissue samples tested. We observed that the tissue samples were easier to homogenize when dewaxed with xylene compared with Citoclear, and therefore used the former reagent for subsequent investigations. Nuclei could be obtained from all tissue samples without proteinase K digestion, although the yield was often reduced when this step was omitted (data not shown). We observed no benefit from cytocentrifugation and used the "spotting" method (see Materials and Methods) for applying cells to slides in most experiments.

Electron Microscopy of Cells Isolated from Paraffin-Embedded Lymphoid Tissue

Transmission electron microscopy of isolated nuclei revealed a range of appearances, from essentially intact cells, through nuclei associated with partially intact cytoplasm, to "naked" nuclei without any cytoplasm (Figure 1). However, closer inspection of some nuclei in the latter category showed that small amounts of cytoplasm remained adjacent to the nuclear membrane. The majority of the nuclei were in the intermediate category (ie, with some cytoplasmic remnants). Nuclear and cytoplasmic membranes showed loss of definition, but intracellular organelles such as mitochondria, Golgi apparatus, and endoplasmic reticulum were often recognizable. Digestion with proteinase K tended to increase the number of naked nuclei and to decrease their morphological preservation.

Immunostaining of Cells Isolated from Paraffin-Embedded Lymphoid Tissue

Good immunostaining of isolated nuclei from lymphoid tissue biopsies (Figure 2) was obtained for a range of markers (Bcl2, CD3, CD5, CD20, CD34, CD79a, Ki-67, Oct2, SLP76, and von Willebrand factor), using both immunofluorescence and immunoperoxidase techniques. Four of the markers evaluated (BOB1, CCND1, CD30, and Pax-5) did not yield satisfactory staining results.

Staining results tended to be best when no proteolytic digestion was used in the preparation of nuclei, although satisfactory staining was often possible with digestion times of up to 20 to 25 minutes, depending on the antibody used. FISH signals were equally good with and without proteolytic digestion, but the intensity tended to weaken after 25 to 30 minutes.

Figure 1. Ultrastructural features of cells isolated from paraffin-embedded tonsil. **a:** A cluster of five cells, all of which appear to be partially or completely intact. Scale bar = 1 μm . **b:** Example of a nucleus with a small amount of attached cytoplasm. Scale bar = 1 μm . **c:** Enlargement of the enclosed area in **b** showing the periphery of the nucleus (N) and rough endoplasmic reticulum (arrowed) within the attached cytoplasm. Scale bar = 0.5 μm . **d:** Two adjacent cells are illustrated, one of which (on the left) is partially intact, containing both endoplasmic reticulum and mitochondria (arrows), whereas the other (on the right) consists of a nucleus surrounded by a very thin rim of residual cytoplasm. Scale bar = 1 μm . **Inset:** Detail showing a mitochondrion. Scale bar = 0.5 μm . **e:** Details of the cytoplasm of a plasma cell showing well-preserved endoplasmic reticulum (ER) and mitochondria (Mi). Scale bar = 0.5 μm .

Flow Cytometric Analysis of Cells Isolated from Paraffin-Embedded Lymphoid Tissue

Flow cytometric analysis of cells isolated from tonsil with antibodies against B-cell (CD20) and T-cell (CD3, SLP76) markers showed labeling of distinct cell populations, as illustrated in Figure 3.

ImmunoFISH Analysis of Cells Isolated from Paraffin-Embedded Tissue

Isolated cells from normal tonsil and various lymphoma types gave good immunoFISH labeling when immunostaining for surface, cytoplasmic, or nuclear markers using the blue fluorochrome Alexa Fluor 350 was followed by conventional FISH labeling. Examples from a range of lymphomas are shown in Figure 4. The immunoFISH procedure clearly showed that anomalies were confined to the neoplastic cell population and absent from non-neoplastic cells. For example, in Figure 4 neoplastic cells (identified by immunostaining for markers such as Ki-67, Oct2, and CD20) from a Burkitt's lymphoma biopsy carry a *c-Myc* translocation, whereas this genetic anomaly is absent from non-neoplastic cells (identified by absence of the tumor-related markers or by immunostaining for CD3).

It was also noted that the presence of a genetic anomaly in more than a single cell lineage could be demonstrated by the immunoFISH procedure. This is illustrated

in Figure 5A, which shows the presence of the *BCR-ABL* fusion gene (associated with the t(9;22) translocation) in cells of myeloid, erythroid, and megakaryocytic lineage in a case of chronic myeloid leukemia.

A "miniarray" slide, on which a number of isolated cell suspensions derived from different specimens were spotted manually, was hybridized successfully for a single antibody/probe combination (Figure 6). It was also easy to place manually 25 well-spaced specimens in duplicate (50 spots) on a single standard glass slide. If a number of different antibody/probe combinations were to be tested by the immunoFISH technique, it would still be possible to accommodate at least eight spots on a single slide without risk of reagent spillover.

Discussion

Reports of FISH analysis on suspensions prepared from paraffin-embedded samples commonly refer to isolated nuclei,^{17,21,25,27,28} but it is evident from the present study that such nuclear suspensions contain many intact or semi-intact cells. This was evidenced by electron microscopic analysis (Figure 1), immunofluorescent labeling (Figure 2), and flow cytometry (Figure 3). Furthermore, immunofluorescent labeling for many cell markers in these isolated cell preparations, both nuclear and extranuclear, survived the FISH procedure. Consequently, it was possible to define simultaneously at the single cell level both cellular lineage/proliferation status and the

Figure 2. Double immunofluorescence labeling of cells isolated from paraffin-embedded tonsil. The different antigens detected are indicated in colors corresponding to the fluorochrome used. All images show blue DAPI nuclear counterstaining. Top row: Many cells are positive for markers of B cells (CD20 and the Oct2 transcription factor) or T cells (CD3 and the adaptor protein SLP76), and there is no overlap between these markers. A plasma cell is recognizable because of its cytoplasmic labeling for CD79a. Bottom row: Immunofluorescence staining for Ki-67 in combination with CD3, CD20, and CD79a allows assessment of the proliferation status of T and B cells. Magnification, $\times 1000$.

configuration of genes studied by the FISH procedure (Figure 4).

This approach has not been reported previously, because immunoFISH techniques have been applied only to tissue sections or to preparations of fresh cells,^{8–13} although two other articles have noted that suspensions prepared from paraffin-embedded tis-

suess contain at least partially intact cells.^{30,31} However, to the best of our knowledge, immunoFISH analysis as described in this article has not previously been applied to cells isolated from paraffin-embedded material. Our experience of the technique we describe (as applied to a range of different biopsies) has indicated that it is robust and reproducible and could be readily adopted in other laboratories. It may be added that the lineage markers we used included signaling molecules (SLP76, LAT) and the transcription factor Oct2, which have been shown in previous studies to be lineage restricted.^{32–37} Surface-associated markers may tend to be lost during cell isolation so that intracytoplasmic/nuclear markers may be particularly suitable for the immunoFISH technique described in this article.

Our study also confirmed that it was usually easier to assess the FISH signal pattern in isolated nuclei than in tissue sections, not only because there was less nuclear truncation artifact but also because problems arising from excessive cellular overlap/cytoplasmic background were less evident (Figure 5B). This is a major advantage of the isolated nuclei technique of the sort described in this article; the majority of nuclei are intact and clearly distinguishable from their neighbors, and consequently the evaluation of complex abnormalities by the FISH procedure (eg, gain or loss of chro-

Figure 3. Flow cytometric analysis of cells isolated from paraffin-embedded tonsils with markers for B cells (CD20) and T cells (CD3 and the adaptor protein SLP76) showing log fluorescence versus cell counts. In the negative control sample, the primary antibody was omitted.

Burkitt Lymphoma

Ki-67. *C-MYC* split-apart Oct2. *C-MYC* split-apart CD20. *C-MYC* split-apart CD3. *C-MYC* split-apart

Follicular Lymphoma

Bcl2. *BCL2* split-apart Ki-67. *IGH* split-apart CD20. *BCL2* split-apart CD3. *BCL2* split-apart

Mantle Cell Lymphoma

CD5. *CCND1* split-apart Ki-67. *CCND1* split-apart CD20. *CCND1* split-apart CD3. *CCND1* split-apart

Diffuse Large B-Cell Lymphoma

Ki-67. *CEP3 CEP7* Ki-67. *BCL2* split-apart CD20. *BCL6* split-apart CD3. *IGH* split-apart

Figure 4. Cells isolated from paraffin-embedded lymphoma biopsies analyzed by the immunofISH technique in which a specific marker is visualized using a blue fluorochrome and gene configuration is assessed using conventional red and green FISH probes. Cells lacking a marker are outlined in red (non-neoplastic cells) or white (neoplastic cells). Note that the neoplastic cells (detected by Ki-67 labeling) in a diffuse large B-cell lymphoma sample (but not the non-neoplastic cells) in the fourth row carry multiple copies of the *BCL2* gene, three copies of the *BCL6* gene, and a split *IGH* gene. Magnification, $\times 1000$.

A Chronic myeloid leukemia

B Mantle cell lymphoma

CCND1-IGH dual fusion. (DAPI counterstain)

C DLBCL

Figure 5. A: Immunofluorescence analysis of cells isolated from a chronic myeloid leukemia trephine biopsy, using dual fusion probes for the t(9;22) translocation involving *ABL* and *BCR* genes. This anomaly is seen not only in myeloid cells (CD15- and MPO-positive) but also in erythroid (glycophorin-positive) and megakaryocytic (LAT-positive) cells. The white outlines indicate marker-negative cells. B: A tissue section and an isolated cell preparation from a case of mantle cell lymphoma analyzed for the t(11;14) translocation using *CCND1-IGH* dual-fusion probes (DAPI counterstain). The background cytoplasmic fluorescence and overlapping/adjacent nuclei in thick parts of the tissue section would render interpretation difficult. These problems are less evident in a thinner part of the section but are essentially absent from the isolated cell preparation. Immunofluorescence labeling of a diffuse large B-cell lymphoma (DLBCL) shows triple copies of *C-MYC* in neoplastic lymphoid cells (circled white) and a normal pattern in an endothelial cell (immunostained by antibodies against von Willebrand factor CD34). Magnification, $\times 1000$.

mosomes or parts of chromosomes) can be more reliably assessed and documented than is possible using tissue sections.

The technique we describe offers a number of other possibilities for future studies. For example, tumors in which neoplastic cells are present in relatively low numbers (eg, Hodgkin's disease) require the use of an immu-

nocytochemical label if FISH procedures are to be performed, and the immunofluorescence analysis of isolated cells would bring the benefit that numerical abnormalities are more easily detected. Furthermore, the ability to define the tumor cell population with an immunocytochemical marker means that it should be possible to distinguish between primary genetic abnormalities, present in all

Figure 6. A: A six-spot cellular “miniarray” of cells isolated from six different paraffin blocks immunostained for the proliferation marker Ki-67 (blue) and probed with a *BCL2* split apart probe (green and red). Ki-67-negative neoplastic cells are outlined in white. There is sufficient separation between each sample to allow immunofISH analysis of each sample with different antibody/probe combinations. **B:** A higher density, 50-spot cellular array for screening multiple biopsies with a single antibody/probe combination. Magnification, $\times 1000$.

tumor cells (eg, *MYC* rearrangement), and secondary changes (present in a subpopulation of tumor cells). Finally, because the immunoFISH analysis of isolated cells allows a genetic alteration to be assigned to a cell lineage (Figure 4), it could be used (using markers such as von Willebrand factor and CD34; see Figure 5B) to confirm the unexpected observation that lymphoma-associated genetic aberrations are often present in endothelial cells.³⁸

The fact that extracted cell suspensions can be stored for long periods before use means that custom nuclear arrays for immunoFISH analysis can be prepared (Figure 6). Tissue arrays are now widely used for evaluating antigen expression or chromosome abnormalities simultaneously in many tumor samples. However, these arrays take time to prepare, and their composition cannot then be changed. In contrast, nuclear arrays can be prepared as and when needed for individual experiments. In the present study, we prepared only small miniarrays of nuclear suspensions, but this approach could clearly be scaled up to allow a genetic anomaly to be studied by the immunoFISH procedure in many samples. It is also possible, provided the spotted nuclear suspensions are adequately separated, to apply a number of different antibody/probe combinations to multiple samples on a single slide.

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