Combined FISH and Immunolabeling on Paraffin-Embedded Tissue Sections for the Study of Microchimerism

BioTechniques 34:242-244 (February 2003)

Fluorescence in situ hybridization (FISH) has proven to be a very useful technique for the study of chimerism and microchimerism (2,4). Based on the detection of sex chromosomes, this technique allows for the identification and localization of donor and recipient cells in cases of sex-mismatched transplantation or cells from mothers and their male fetuses in studies of fetomaternal cell trafficking. For the further assessment of chimerism, the combination of FISH and techniques of protein detection allows for the determination of the phenotype of persistent chimeric cells. However, such combined techniques have proven to be difficult to apply to paraffin-embedded tissue specimens, which is the common preparation of tissue samples following biopsy, surgery, or autopsy.

To date, studies of chimerism using simultaneous FISH and protein detection have focused on cytoplasmic proteins such as albumin and cytokeratin (1) or p53 (5). Methods for the identification of cells by sex chromosome constitution and the presence of antigens with a low level of expression or cell-surface molecules such as cluster of differentiation markers have been performed sequentially on a single tissue section or on thin serial sections. This approach requires a search for co-localized FISH and immunolabeling signals in two separate experiments, necessitating considerable microscope and image-capturing time to identify target cells. Artifacts and background can confound the visualization of DNA and protein signals in the same cell generated from two separate experiments. In addition, the localization of a cell by FISH in one tissue section and the determination of its phenotype by immunolabeling on a serial section either above or below the original section, which relies on the superimposition of

Table 1. Introducing Different Immunolabeling Procedures in the FISH Protocol

FISH Steps ^a	Peroxidase ^b	Fluorescence ^c
Deparaffinization-rehydration Steps 1–4	+: CD45, CD34, CK ^d	Negative for all antibodies tested
Acid-Heat treatment Steps 5–8	+: CD45, CD34, CK ^d	Negative for all antibodies tested
Protease digestion Steps 9–12	Not done	Not done
Hybridization Steps 13–17 and day 2 washes	+: CK, not done for others.	+: CK, negative for CD34 and CD45

^aAs described in Johnson et al. (3). Each row represents the immunolabeling being performed after the FISH protocol step indicated in column 1. For example: Acid-Heat treatment steps 5–8 (row 2); the immunolabeling procedure was performed after step 8 and before step 9 of the FISH procedure.

^bImmunoperoxidase staining was performed using the Envision+ kit (Dako, Carpinteria, CA, USA) with the aminoethylcarbazole substrate.

^cFor immunofluorescence staining, tissue sections were washed in PBS, incubated with 10% normal goat serum (Jackson ImmunoResearch, West Grove, PA, USA) for 20 min, rinsed in PBS, and then incubated with the primary antibody for 1 h. Following three washes with PBS for 1 min each, the sections were incubated with a phycoerythrin labeled goat-anti mouse immunoglobulin (BD Biosciences, San Jose, CA, USA) for 30 min. The sections were then washed three times in PBS for 5 min each and further processed by FISH.

^dEthanol dehydration (step 13 in FISH protocol) was removed to avoid loss of peroxidase stain.

+: Stain easily recognizable by fluorescent microscopy.

CK, cytokeratin, AE1/AE3 anti-cytokeratin antibody (Chemicon International, Temecula, CA, USA); CD45, common leukocyte antigen, anti-CD45 antibody (Dako); and CD34, Qbend/10 anti-CD34 antibody (Signet, Dedham, MA, USA).

two separate images to approximate co-localization, can be highly subjective.

We previously reported a versatile method to perform FISH on a variety of paraffin-embedded tissues for studies of microchimerism (3). This protocol can be broken down into groups of processing steps, as shown in Table 1. We introduced the immunolabeling techniques at different points in the FISH procedure (i.e., between groups of FISH processing steps) to evaluate the quality of the FISH and immunostaining signals simultaneously. Immunofluorescence staining failed for all antigens when incorporated before the protease digestion (between FISH steps 4 and 5 or 8 and 9). After protease digestion, only cytokeratin could be stained (Figure 1E). Immunoperoxidase staining was successful for all antibodies (Figure 1, A–C), provided that the ethanol dehydration step (step 13 in

FISH protocol) was removed. Though the peroxidase stain using aminoethylcarbazole is typically detected with a light microscope, it was also visible through single-bandpass red and dual-bandpass filters with the fluorescent microscope. The peroxidase staining was more intense if it was performed after rather than before the acid and heat treatment in the FISH procedure (i.e., between FISH steps 8 and 9). The immunoperoxidase staining between steps 8 and 9 of the FISH procedure seems to be more widely applicable and generates a stronger stain; thus, it is the protocol we recommend to use. For both immunoperoxidase and immunofluorescence, no staining was obtained with isotypic control mouse IgG1 (Figure 1, D and F). All procedures were performed on human skin, liver, thyroid, and brain tissue.

While immunoperoxidase staining

Figure 1. Photomicrographs showing interphase FISH combined with immunostaining. (A and B) Immunoperoxidase staining of CD45 (small arrows, dark red) and X chromosomes by FISH (large arrow) in cells from a liver section. Panel B demonstrates that the detection of CD45 in two cells (small arrows) is possible at low magnification (400 $\times$), whereas the detection of X chromosome signals must be confirmed at higher magnification (1000 $\times$), as shown in panel A (large arrow). (C) Immunoperoxidase staining of CD34 (small arrow, orange-brown) and X chromosomes by FISH (large arrow) on a skin section lining the endothelium of a small blood vessel (1000 $\times$). (D) Mouse IgG1 isotypic control and X chromosomes by FISH (large arrow) on a skin section showing lack of peroxidase staining (400 $\times$). (E) Immunofluorescence staining of cytokeratin (small arrow) and X chromosomes by FISH (large arrow) showing intensely stained keratinocytes from a skin section (1000 $\times$). (F) Mouse IgG1 isotypic control and X chromosomes by FISH (large arrow) on a skin section showing lack of immunofluorescent staining (400 $\times$). In all panels, nuclei are counterstained with DAPI (blue), and X-chromosome probes are fluorescently labeled with Cy3 (light red). Fluorescent signals representing X chromosomes are not seen in all nuclei, as all signals were not within the plane of focus of images at 1000 $\times$ magnification or were not visible at 400 $\times$ magnification. For immunofluorescence, cytokeratin and mouse IgG1 isotypic control secondary antibodies were labeled with phycoerythrin (bright red). For immunoperoxidase, CD34 and CD45 antibodies were detected with aminoethylcarbazole substrate (appears as dark red, orange, or brown).

was successful when incorporated at different locations in the preexisting FISH protocol for the antibodies that were tested, immunofluorescence staining proved to be more challenging. Our results suggest that antibody digestion can occur when the immunofluorescence staining is performed before the proteinase K step in the FISH procedure. However, the peroxidase detection system allows for the staining of all antigens that were tested here when incorporated before the proteinase K step. This is most likely because the stained complexes that are produced by the peroxidase reaction are not sensitive to proteinase digestion, unlike fluorescent-based complexes. Regardless of the staining system that is used, the acid and heat treatments in the FISH protocol serve as the required pretreatment for immunolabeling. This simplifies the immunostaining portion of the combined procedure without compromising the quality of the final results.

Our study is novel in that we compared different protocols of staining, allowing for the detection of multiple antigens including surface markers on a variety of paraffin-embedded tissue sections. This is an important method for studies of microchimerism, as it remains to be shown if the persistent cells are terminally differentiated blood cells, lymphoid precursor cells, or another cell type (e.g., endothelial cells). This technique improves on previously published procedures that focused on the detection of highly expressed antigens such as albumin and p53 in a single tissue type.

In conclusion, we have developed a versatile staining procedure combining FISH and immunolabeling, which is a powerful tool to localize and characterize the phenotype of chimeric cells following transplantation, transfusion, and fetomaternal cell trafficking associated with pregnancy. In addition, this technique may have widespread applications for the detection of cytogenetic abnormalities in phenotypically distinct cells in areas of research such as oncology or hematology.

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Dr. Khosrotehrani was supported by the "Bourse Lavoisier du ministère des affaires étrangères", France. We thank Pr Selim Aractingi and Caroline Ledanff for helpful discussions on immunofluorescence techniques. Address correspondence to Dr. Kirby L. Johnson, Division of Genetics, Department of Pediatrics, Tufts-New England Medical Center, Box 394, 750 Washington Street, Boston, MA 02111, USA. e-mail: kjohnson@lifespan.org

Received 3 October 2002; accepted 11 December 2002.

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Shotgun Library Construction in a Day

BioTechniques 34:244-250 (February 2003)

The generation of random genomic libraries is an important component of several key technologies, including shotgun sequencing of genomic DNA (2) and genomic SELEX for isolation of protein binding ligands (4). We are particularly interested in the generation of random libraries for expression library immunization (ELI), a technique that screens genomic libraries for DNA fragments that protect animals against infection from the pathogen in question, thus providing leads for vaccine candidates (1). Traditionally, random genomic libraries have been constructed using DNA that has been fragmented either by physical shearing (such as nebulization or sonication) or by partial digestion with a restriction enzyme. However, there are a number of practical disadvantages associated with these methods: (i) they are time consuming, and (ii) they require large amounts of DNA (e.g., both ELI and SELEX require 25 µg genomic DNA as the starting material). This high demand is driven by DNA loss during size fractionation and the relatively inefficient processes of blunt-end ligation or of using adaptors for cloning (6). A protocol that uses amounts of genomic DNA in the nanogram range has been described (3), but this method still has the disadvantage of being fairly labor intensive and lengthy.

We have developed a rapid and facile protocol for constructing genomic libraries from nanogram amounts of DNA. The first part of the technique for producing insert DNA uses a similar principle to one previously described (4). The DNA is initially amplified using oligonucleotide primers that have two distinct parts: a sequence of random nucleotides that is used to prime the genomic DNA and a fixed sequence of nucleotides that is common to every oligonucleotide in the mixture [5'-CC-UCGCGUCGUCUG(N)₉-3', where underlining denotes the constant region]. The average product length is influenced by the length of the random region of the template, as well as by the

annealing temperature. We empirically determined that a length of nine random nucleotides and an annealing temperature of 25°C resulted in the production of DNA fragments within the 300–800 bp size range, which was optimal for constructing libraries for ELI. To amplify the DNA, a 20-µL reaction was set up with 10–100 ng genomic DNA that had been purified by standard methods, 2 µL Klenow 10× buffer, 0.3 mM dNTPs (Promega, Madison, WI, USA), and 50 ng random primers (synthesized on an ABI 394 DNA/RNA Synthesizer; Applied Biosystems, Foster City, CA, USA). To generate the first strand of DNA, the mixture was first heated at 93°C for 3 min, chilled on ice for 2 min, and treated with 5 U Klenow enzyme (Promega). After a 5-min pre-incubation stage on ice, the tube was transferred to a thermal cycler, where it was subjected to incubations of 25 min at 25°C and 5 min at 50°C. The reaction was then heated at 93°C for 2 min and chilled on ice for 2 min. To synthesize the second strand of DNA, an additional 5 U Klenow were added to the reaction, which was then subjected to the same series of incubations as described above. To remove the random primers and unincorporated nucleotides, the reaction was passed over a gel filtration column (Edge Biosystems, Gaithersburg, MD, USA) and centrifuged for 2 min at approximately 360× g to produce an eluate that contained the purified dsDNA product. Interestingly, we did not observe a visible difference in the first Klenow step whether (i) the DNA was nebulized or untreated, or (ii) we used 10 or 100 ng starting material (not shown). We did not test below the 10-ng range of DNA.

To produce sufficient amounts of DNA for cloning purposes, this product provided the template in a subsequent PCR step that used a primer corresponding to the constant region of the random primer mixture used for the initial amplification step (5'-CC-UCGCGUCGUCUG-3'), since this complemented one of the two template strands. In addition, this primer contained five UTP residues required for subsequent linkage of the PCR products (see below). PCR was carried out in a 100-µL volume containing 2 µL purified dsDNA product generated by