

The combination of SKY and specific loci detection with FISH or immunostaining

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Abstract. Spectral karyotyping (SKY) represents an effective tool to detect individual chromosomes and analyze major karyotype abnormalities within an entire genome. We have tested the feasibility of combining SKY and FISH/protein detection in order to combine SKY's unique abilities with specific loci detection. Our experimental results demonstrate that various combined protocols involving SKY, FISH and immunostaining work well when proper procedures are used. This

combined approach allows the tracking of key genes or targeted chromosome regions while monitoring changes throughout the whole genome. It is particularly useful when simultaneously monitoring the behavior of both protein complexes and DNA loci within the genome. The details of this methodology are described and systematically tested in this communication.

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Spectral karyotyping (SKY) represents a multicolor whole genome painting approach to karyotype analysis. It is fast becoming the method of choice especially in the field of cancer cytogenetics where chromosome aberrations frequently occur (Schrock et al., 1996; Lengauer et al., 1998). The power of SKY to monitor major chromosome changes within a single hybridization experiment provides unmatched rapidity and precision for karyotype analysis. This whole genomic approach serves as an effective quick-look screening tool that provides the information needed to focus on identifying numerous structural abnormalities and marker chromosomes. Since its introduction in 1996, SKY as well as M-FISH (multicolor-FISH) have been used extensively for karyotype analysis in both human and

mouse cell lines with normal or mutant characteristics (Liyanage et al., 1996; Speicher et al., 1996; Ried et al., 1998).

In general, locus specific FISH is often used to confirm results generated by SKY with known probes (Padilla-Nash et al., 1999; Adeyinka et al., 2000). FISH can further define altered regions by using the combinations of known probes (Heng et al., 1997). This type of confirmation has been done on chromosome slides that have not been subjected to SKY processing but from the same preparation used for SKY. To date, separate SKY and FISH procedures work well in most laboratories.

There are three circumstances that have triggered our interest in developing these combined procedures for SKY and FISH co-detection. First, the limited availability of clinical samples has forced us to perform FISH on slides that have been subjected to SKY processing in order to confirm the SKY results. Second, we often have had difficulty identifying the host chromosome when performing FISH analysis due to extensive karyotype changes within the host genome. The banding patterns were equivocal and misleading and we have had to rely on SKY analysis for identification of the host chromosome. Finally, during the process of studying mouse meiotic chromosomes with SKY, it became evident that visualizing the synaptonemal complex (SC) protein would provide an impor-

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tant protein marker for the tracing of the various stages of prophase. It would enhance the information quality of these tests, if SKY and SC detection could be combined. Thus we have tested procedures to combine FISH/immunostaining with SKY.

The idea of combining SKY with locus specific FISH and immunostaining for protein, is a logical extension of the SKY methodology. In this report, we describe four protocols that use variations of the combination of single locus FISH and immunostaining with SKY. These protocols are the following: SKY detection on previously FISHed slides, FISH detection on previously SKYed slides, FISH/SKY simultaneous detection on unprocessed chromosome slides and finally, SKY detection on immunostained slides. The indications and applications for each of these protocols are systematically examined. In addition, key elements to successful FISH or SKY applications on previously used chromosome slides are analyzed.

The successful use of various protocols demonstrates that this new combination is an efficient tool for human and mouse chromosome research, especially for the karyotype analysis of human cancer cell lines.

Materials and methods

Human and mouse chromosome preparations

Human and mouse lymphocytes were used, hypotonic treatment applied, fixation with methanol:acetic acid (3:1) and then chromosome spreads were prepared and air-dried (Heng and Tsui, 1993).

Mouse meiotic surface spreads

Approximately 10 μ l of resuspended testicular cells were touched to a 10 mM NaCl hypotonic solution and the cells were then picked up on a glass slide. The cells were fixed in freshly prepared 2% paraformaldehyde with 0.03% SDS at a pH of 8.2, then washed in 0.4% Kodak PhotoFlo 200 three times for 1 min. They were then briefly dried and used immediately for SKY or stored at -20°C for later use (Heng et al., 1994, 1996).

SKY/FISH and SKY/immunostaining co-detection methodology

SKY after FISH. To test this protocol, slides previously analyzed by FISH detection were subjected to SKY. Both direct-labeled FISH and indirect labeled FISH slides were used to compare the outcomes after SKY detection. For direct-labeled FISH, FITC-dUTP was used for probe labeling. For indirect labeled FISH, probes were labeled with biotin then detected by FITC-avidin (this also includes an amplification step). These procedures were done according to standard published protocols (Heng et al., 1992). After quickly examining the FISH results with fluorescent microscopy, slides with clear FISH signals were selected for SKY analysis.

Previously FISHed slides were cleared of oil and the coverslip was removed by washing in $2 \times \text{SSC}$. Slides were then denatured in 70% formamide, $2 \times \text{SSC}$ at 72°C for 2 min and then immediately placed in a series of cold 70%, 80% and 100% ethanol (2 min each). The SKY-paint mixture was denatured at 80°C for 7 min followed by incubation at 37°C for 1 hr. Hybridization was performed by loading 10 μ l of denatured SKY-paint onto the denatured slide. After sealing the edges of the coverslip with rubber cement, the slides were transferred into a humidified chamber and incubated at 37°C for 24–36 hr.

To perform detection, slides were then washed with 50% formamide in $2 \times \text{SSC}$ for 5 min, $1 \times \text{SSC}$ two times for 5 min each and then $4 \times \text{SSC}$ with 0.1% Tween 20 for 2 min. Signals were detected as recommended by the manufacturer. The slides were then washed, air-dried, then stained with DAPI and mounted with Antifade (Liu et al., 2000).

The overall quality of SKY hybridization was estimated by examining the color pattern of the SKY signal. Twenty mitotic figures of high quality color and signals from previous FISH analysis were captured using a CCD camera.

FISH after SKY. In this procedure slides that were used previously for SKY were further utilized for FISH detection. The protocol for SKY detection is as described above. Slides were examined by microscopy to ensure that bright, defined multi-colors were present indicating high quality SKY detection. After clearing the oil and a brief washing of the slides with $2 \times \text{SSC}$, the samples were re-denatured in 70% formamide, $2 \times \text{SSC}$ at 72°C for 2 min followed by a graded series of cold ethanol (70%, 80% and 100%). To prepare FISH probes suitable for SKY co-detection, the same DNA probe has been labeled with biotin-dATP, FITC-dUTP and tetramethyl-rhodamine-dAUP respectively, for the purpose of comparing direct-labeled FISH detection and indirect labeled FISH detection on previously SKYed slides. These different labeled probes were denatured then hybridized to their respective re-denatured slides.

The procedure for FISH detection was as described. Following overnight hybridization at 37°C , slides were washed using the standard FISH protocol. Specifically for fluorescent direct-labeled probes when no detection and amplification was used, slides were examined following the wash steps. For biotin labeled probes, detection with FITC-avidin and amplification were both used and then the probes were examined by microscope (Heng and Tsui, 1993).

FISH with SKY. This procedure is used when performing simultaneous detection of locus specific FISH and SKY. Unlike the other protocols, the use of fresh unprocessed chromosome slides was essential for simultaneous SKY and FISH detection. The protocol for slide treatment, hybridization, washing and detection was performed according to conventional SKY standards except for the step that follows denaturation and prehybridization involving the mixing of the FISH probe (50 ng) with SKY-paintTM. For the comparative study, this step was varied by using different FISH probes (direct or indirect labeled) along with SKY probes to determine the most effective FISH probe on a particular SKY background. Direct-labeled FISH probes (labeled with FITC or rhodamine), or commercially available probe (LSI, AML, SO/TEL, SG; Vysis Inc., Downers Grove, IL) and indirect labeled FISH probes (labeled with biotin) were then used.

A methodology difference occurred between the two types of FISH probes that should be noted. When direct-labeled FISH probes (labeled with FITC or rhodamine) are used and combined with SKY, the procedures for washing and detection are the same as when performing SKY alone. When indirect-labeled FISH probes (labeled with biotin) are used and combined with SKY, an additional interval of FITC-avidin incubation was necessary to visualize the FISH probe following SKY detection.

SKY after immunostaining

Surface spread meiotic slides were used for antibody detection (anti-core, anti-syn, and anti-centromere) (Dobson et al., 1994; Heng et al., 1994). Slides were washed, then dried and examined under microscopy to ensure the success of the antibody detection. Slides were then ready for SKY detection as described above in section 'SKY after FISH'. Longer hybridization time (3–4 d) is needed for surface spread meiotic slides.

FISH detection on FISHed slides

To compare the quality of FISH redetection using FISHed slides, the same probes from chromosome 21, 12, 7 and X were labeled by direct labeling and indirect labeling. Two chromosome slides were denatured and incubated with direct labeled and biotin labeled chromosome 12 cosmid probes respectively. Essentially, for the direct-labeled probe, no FITC-avidin or amplification was required. After hybridization, washing and detection, slides were examined microscopically. These slides were then subjected to a second FISH analysis using probes from chromosome 21 (direct labeling and biotin labeling). The direct labeled and biotin labeled probes were hybridized to their respective previous direct labeled and biotin samples. After hybridization, washing and detection, slides were examined under the microscope. The third FISH analysis used probes from the X chromosome, and the fourth analysis of FISH used probes from chromosome 7.

Image acquisition and analysis

Spectral analysis was performed using software developed by Applied Spectral Imaging. Chromosomes were karyotyped according to color and size following the editing of the chromosomal contours. Images were assembled in PowerPoint then printed using a Kodak color printer.

For SKY combined with protein immunostaining a brief modification for image acquisition was needed. The SC image was acquired using the SKY

filter (not the DAPI filter) after acquiring the SKY image, in order to obtain consistent and better quality images of the SC. This is accomplished first by capturing the color of the protein-stained image then setting the option "DAPI" for picture acquisition. The DAPI filter is not employed as required for standard SKY images but the same filter is used that was used in the spectral color image. The second black and white image is then captured. This will produce superior quality images of SKY combined with protein immunostaining.

When FISH signals are not strong due to the small size of a FISH probe or lack of amplification, it is essential that the modified procedure mentioned above is followed in order to record the FISH image. Since the signals of FISH are visible when the SKY filter is used, the acquisition of the FISH image by using the DAPI option and SKY filter will generate black and white images of chromosomes with stronger FISH signals. Changing image brightness and contrast can also enhance the FISH signals.

Results

Four different protocols have been examined so far and all the tested procedures had useful and practical applications when proper combinations were used. The combinatorial techniques show great promise especially in the area of studying a specific gene or particular chromosome region while at the same time monitoring overall genomic changes. In the following section the useful applications of each technique, including solutions to glitches and protocol limitations are reviewed.

SKY detection on FISHed slides is successful for acquiring both the SKY image and FISH signals

It was a concern that the SKY treatment would remove the FISH signals following additional rounds of denaturation and extensive washes. This was indeed the case when direct-labeled FISH slides were used for SKY. For all tested slides, the results were the same regardless of the color of the direct-labeled FISH probes. The protocol using direct-labeled FISH slides for SKY is therefore not successful for SKY-FISH co-detection due to the loss of previous FISH signals. In order to solve the problem of direct-labeled probes being removed during denaturation, we tested indirect labeled probes with antibody amplification. The principle feature in this design is that the antibody-antigen complex might be more stable providing resistance to re-denaturation. In fact, it is this complex that provides the basis for DNA-probe in situ co-detection (Heng et al., 2000). This combination of SKY and indirect FISH proved to be effective and the previous FISH signals were well maintained following SKY detection.

Certain features including the age of previously FISHed slides, the size of the FISH probe and the additional amplification step influence the outcome of SKY/FISH co-detection. To ensure the best quantity of the FISH signal after SKY detection, it is necessary to check the intensity of the FISH signal before performing SKY. Fresh FISH slides usually contain strong FISH signals. When using previously FISHed slides, it is necessary to check slides under a microscope to determine if the FISH signals remain visible. If the signals are visible, then they are acceptable for co-detection with SKY. We have used FISHed slides stored for over 6 months in the dark at -20°C , and both the FISH signals and SKY painting retained good coloration.

When the protocol of SKY after FISH was used, occasionally the FISH signals were too strong due to the large sized probe interfering with the SKY image. When the FISH signals were too bright, the overall color of the SKY image could dramatically change its tone. This can lead to the possible misidentification of chromosomes. Therefore, moderate FISH signals are best suited for superimposed SKY analysis since they minimize interference with the actual SKY image color. When this criterion was satisfied the SKY-view program could accurately identify chromosomes (Fig. 1).

Additionally, increased background intensity will lower the quality of SKY detection. Avoiding antibody or other reagents that tend to increase the background minimizes this problem. SKY color is also improved when FISH probes are detected without amplification since amplification usually increases the background along with the specific FISH signals. In general, however, amplification will not dramatically interfere with the color of subsequent SKY. When smaller probes are used for SKY/FISH co-detection signal amplification is recommended for generating strong FISH signals on SKY painted chromosomes.

To acquire the FISH signals, it is necessary to use the "DAPI" option with the SKY filter. Fig. 2 demonstrates the value of this adjustment, especially when the FISH signals are weak. By adjusting the brightness and contrast, the FISH signals are clearly displayed.

This protocol has been used for the successful identification of insertion sites on host chromosomes for transgenic mice as well as for the mapping of genes in cell lines with altered karyotypes. Chromosome identification is always a challenge when mapping transgenes on specific mouse chromosomes because it is very difficult to distinguish between different mouse chromosomes with similar morphological features. To prove the practicality of using this protocol we have randomly tested ten independent transgenic mouse lines with insertions on various chromosomes. The results were excellent with successful identification of the host chromosomes in all ten lines. We anticipate that this protocol should be extremely useful when tracing integration sites of transfected fragments within a tumor cell line, considering the fact that these cell lines often consist of scrambled chromosomes that are difficult to identify when compared to a normal karyotype.

FISH detection on previously SKYed slides: a partial success

Most chromosome changes detected by SKY are then usually confirmed by FISH. Accordingly, a protocol using FISH detection on previously SKYed slides would be advantageous and practical when defining these altered regions identified by SKY. Unfortunately the color pattern imposed by SKY detection no longer exists following the ensuing FISH processing. The failure of this protocol is due to two technical limitations. The first is the interference between the biotin-avidin interaction and its related antigen-antibody detection during SKY and indirect FISH. The second is that the direct-labeled SKY probes are easily removed during re-denaturation that is part of the subsequent FISH detection.

The interference from the overlapping uses of biotin-avidin during SKY and indirect FISH co-detection causes a dramatic

Fig. 1. Examples of SKY detection on previously FISHed slides. **(A)** Mouse gene mapping using the SKY-FISH co-detection approach. FISH signals are indicated by arrows while the SKY color indicates that chromosome 9 is the host chromosome for this gene. **(B)** Identification of the host chromosome in transgenic mice. Indirect FISH without amplification detected the transgene and the SKY color indicates that this transgene is integrated into chromosome 4.

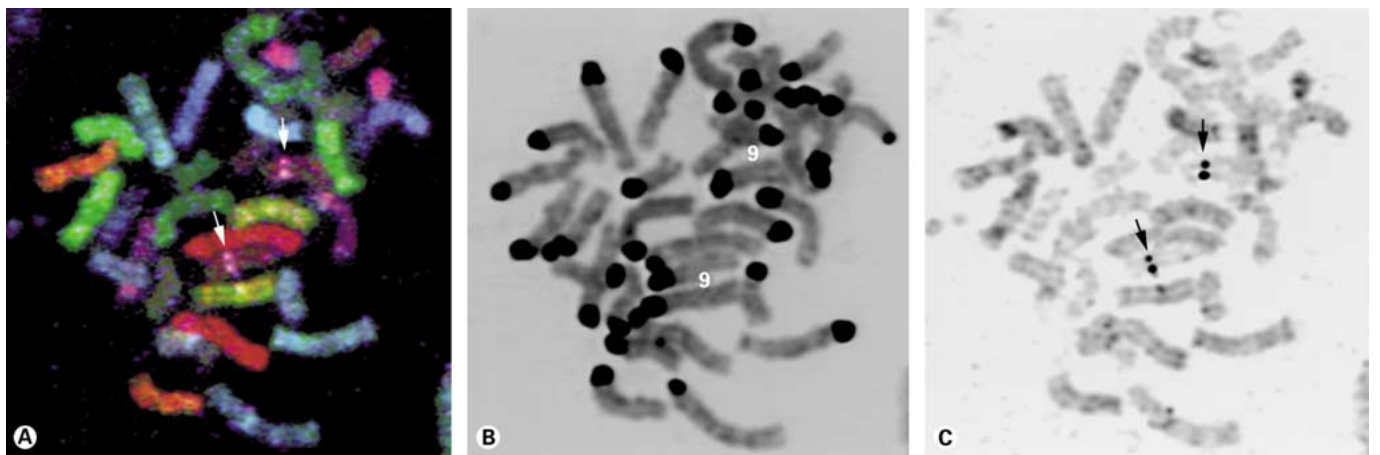
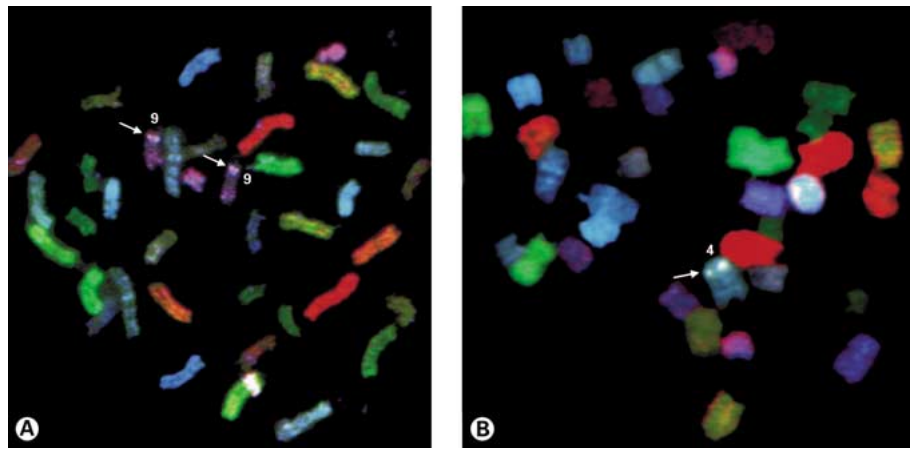


Fig. 2. An example of FISH signal acquisition. When FISH signals are not very strong, it is useful to apply the “DAPI” option to capture the FISH image. **(A)** SKY and FISH co-detection image. Note that the FISH signal is not very clear. **(B)** The DAPI image with the DAPI filter. No FISH signal is observed. **(C)** Image of the DAPI option with the SKY filter. The FISH signal can be clearly seen after adjusting the brightness.

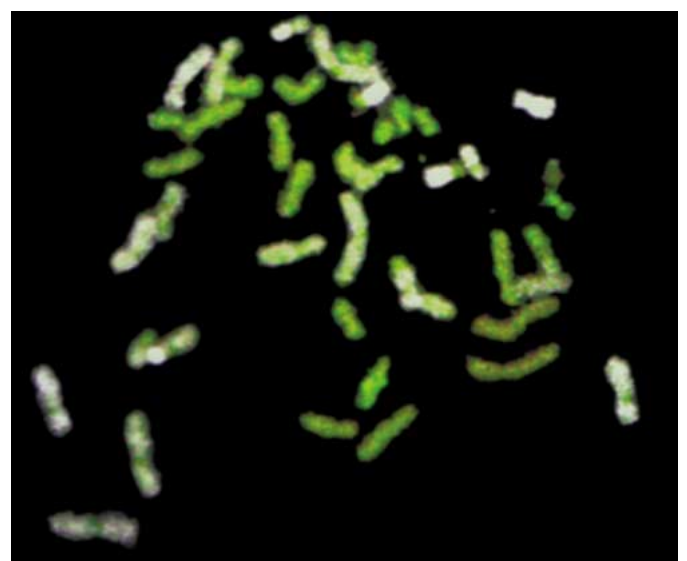


Fig. 3. An example of color interference from FISH on SKYed slides. Note that the only SKY colors remaining are yellow, white and yellow-green colors.

color alteration for SKY. This occurs when conventional FISH detection with an indirect labeled probe (labeled with biotin, detected by avidin and tagged with FITC) is used. Almost half of the chromosomes display a strong FITC color thus making microscopic detection of either FISH or SKY signals difficult. The current SKY system uses Cy5 strep avidin and when FITC-avidin is used for FISH detection, those chromosomes labeled with Cy5 will be enhanced. The FISH signal is not discernible on those chromosomes with the FITC background. Only with probes that are FITC negative staining can visualization of the FISH probe by FITC be possible. Even in this case, SKY based hybridization is not effective. All chromosomes have a yellow, white and yellow-green color after image acquisition and SKY-view analysis. Therefore it is not practical to use an indirect FISH detection system at this time (Fig. 3). To avoid interference from avidin and antibodies involved in both SKY and FISH (Cy 5.5 is tagged with an anti-mouse antibody in the SKY system), we elected to use direct fluorescent-labeled FISH probes. When a direct-labeled probe is used without FITC-avidin detection and antibody amplification, FISH is successful; however, the color of the SKY hybridization is still lost during redensaturation (data not shown).

The second technical problem involving direct-labeled SKY probes is that they are easily removed during redensaturation in subsequent FISH detection. This reflects the fact that three of SKY detection's five main fluorochromes (rhodamine, Texas-red and FITC) utilize direct labeling. These direct-labeled fluorochromes do not survive the redensaturation process thus resulting in a deficiency in the SKY detection.

Our experiments reveal that when FISH detection is performed on previously SKYed slides the SKY coloration fades, therefore the SKY-FISH co-detection is not successful. Despite the fact that the SKY color does not remain after redensaturation, the direct-labeled FISH detection works fine and still provides a useful method to confirm SKY results. Using this protocol, we have confirmed the involvement of mouse major satellite sequences in a SKY karyotyped tumor cell line. According to our experience SKYed slides aged from one week to twelve months kept in the dark at -20°C can be used for successful direct FISH to confirm SKY results.

*Detecting a specific locus within the whole genome:
FISH and SKY simultaneous detection*

The ideal method that avoids the redensaturation step and saves time is simultaneous detection with SKY and FISH. We have successfully used direct-labeled fluorescent probes that do not require any antibody for the FISH probe detection and will not interfere with SKY. As expected, both SKY and FISH images were excellent and there was no color interference. Signals from FISH probes appear as two defined dots on one pair of chromosomes representing each sister chromatid. The SKY patterns and the classification of chromosomes by SKY-view are as expected. No difficulties were encountered in the identification process following the defining of the contours for SKY or when performing FISH mapping. Examples are presented here using commercially available direct FISH probes for FISH-SKY co-detection based on this protocol (Fig. 4).

In theory, to combine FISH mapping and SKY the color of the direct-labeled FISH probe should contrast with the color of SKY painted chromosomes. For probes with known chromosome locations, we can selectively choose a fluorescent tag (either FITC, rhodamine or Cy 3) that will contrast against the SKY painted background. When mapping probes on human chromosome 8 (this chromosome is detected by FITC for SKY) for example, it is best to avoid labeling the probe with FITC. Similarly, when mapping probes on chromosome 20 it is best to avoid rhodamine and on chromosome 14 the use of Texas-red should be avoided. Finally, when mapping probes on chromosomes 17 and 2, avoid the use of Cy 5 and Cy 5.5 respectively. For mapping probes on the remaining chromosomes, either FITC or rhodamine will work well since neither color blends with the SKY painted chromosome color. Similarly, for mouse gene mapping and SKY co-detection, FITC should be avoided for chromosome 13 mapping and rhodamine should be avoided for chromosome 16 mapping.

When using probes with unknown chromosome locations, one can label a probe with a mixture of a two-color tag (i.e., FITC and rhodamine). This ensures that one of the colors of the FISH signal will be visualized and contrast with the SKY painted chromosome color. For example, if a gene was located on chromosome 20 (this chromosome is labeled by rhodamine in the SKY system), the rhodamine labeled portion of the probe would not be easily visualized, while the FITC labeled portion of the same probe (which is the opposite color) will be visualized. Similarly, if the gene were located on chromosome 8 (this chromosome is labeled by FITC in the SKY system) the FITC portion would not be easily visualized, however, the rhodamine portion would be easily seen.

Interestingly, in contrast to the above theory, the FISH signals of large probes usually can stand out from the SKY painted background even when a similar color was used for both FISH and SKY. This is especially true when performing SKY after FISH. In this case, the biotin labeled/FITC-detected FISH probe can easily be distinguished from FITC-painted background generated by SKY. It is likely that certain combinations would generate less optimal results. More comprehensive research is needed to search for the ideal color combinations or new fluorochromes for FISH and SKY co-detection.

One final consideration of SKY and FISH co-detection is to distinguish the types of signals during analysis. To identify translocations, one should expect to detect color changes across the width of the arm of a chromosome. This is differentiated from FISH probes by visualizing two sets of colored "spots" representing two sister chromatids. It is easy to distinguish translocations from FISH signals except when the FISH signals are too bright and overlap creating a band across the width of the chromosome simulating a translocation.

The combination of protein detection and SKY is specifically advantageous when studying DNA-protein interactions

With the success of simultaneous SKY and FISH co-detection, we were encouraged to expand this concurrent detection method for use with protein. This was achieved by combining protein immunostaining with SKY painting. To avoid color

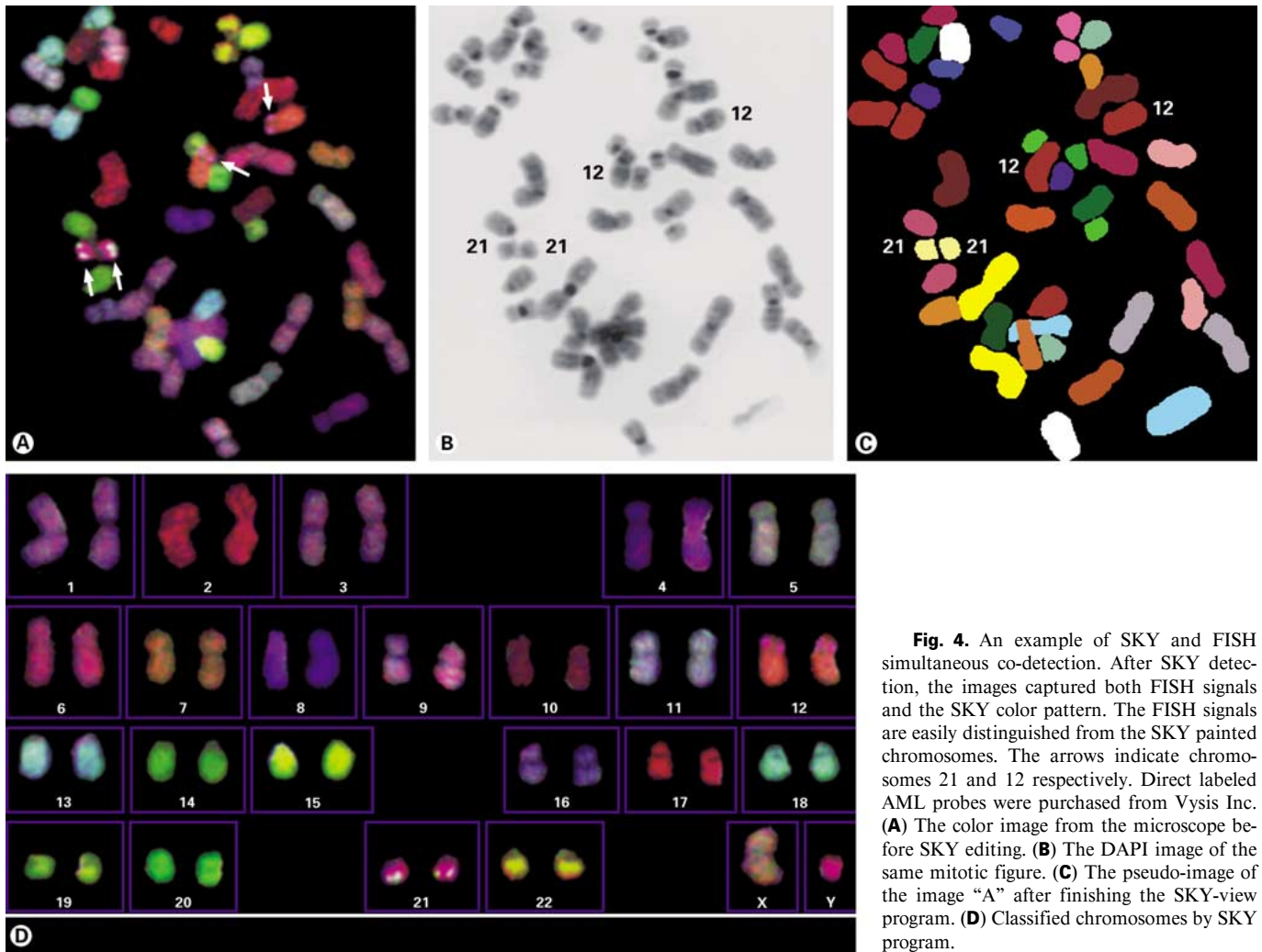


Fig. 4. An example of SKY and FISH simultaneous co-detection. After SKY detection, the images captured both FISH signals and the SKY color pattern. The FISH signals are easily distinguished from the SKY painted chromosomes. The arrows indicate chromosomes 21 and 12 respectively. Direct labeled AML probes were purchased from Vysis Inc. **(A)** The color image from the microscope before SKY editing. **(B)** The DAPI image of the same mitotic figure. **(C)** The pseudo-image of the image "A" after finishing the SKY-view program. **(D)** Classified chromosomes by SKY program.

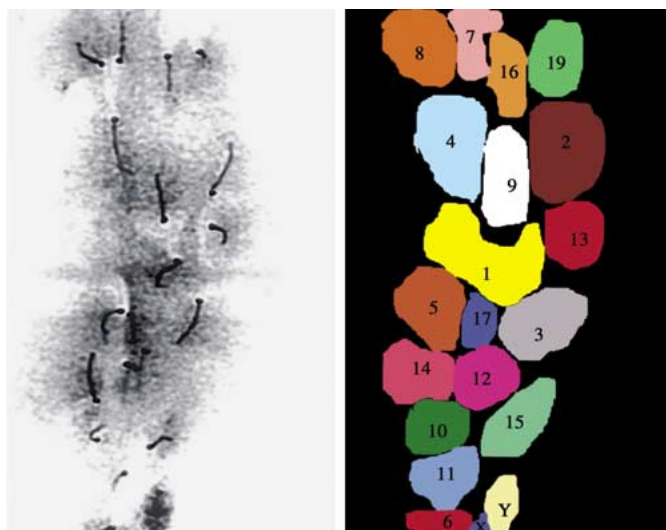


Fig. 5. An example of SKY and protein co-detection on mouse meiotic chromosomes. SC proteins were detected by SC antibody with FITC (left panel), while each chromosome was colored by SKY-paint for specific identification (right panel). The SC protein can be used as a marker for the particular stage of meiosis and the pairing behavior can be monitored by the SKY color.

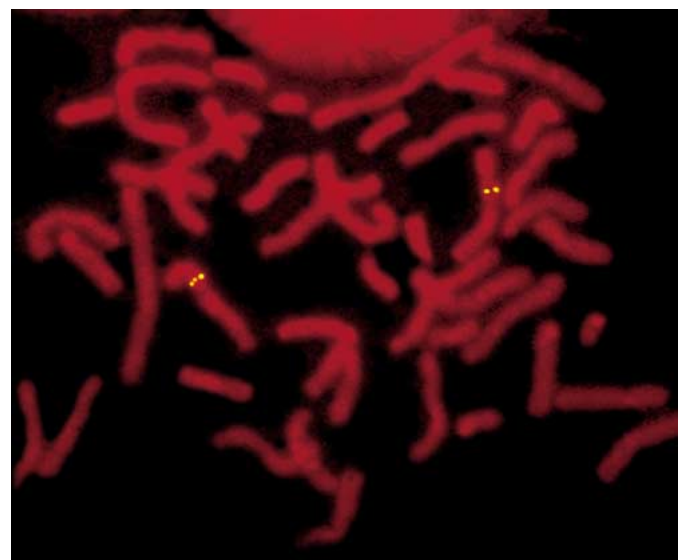


Fig. 6. An example of FISH detection on a previously used FISH slide. The first three FISH detections used a direct-labeled probe. Following successive denaturing no previous FISH signals are apparent. By contrast, if one of the first three FISH detections were achieved by indirect FISH, then that FISH signal would remain creating background interference for any subsequent FISH.

Table 1. Comparison of protocols and expected results

Sequence of protocols	Expected results	Applications	Primary methodology concept
FISH after SKY	Only FISH	Confirm SKY data, define regions detected by SKY	Use fluorescent direct labeled probe for FISH
SKY after FISH	Both FISH and SKY	Gene mapping – for mouse and cancer cell lines host chromosome identification for transgenic mice and ES cell lines	Use indirect labeled probe for FISH
	Only SKY	Re-examine FISHed slides	Use direct labeled probes for FISH
FISH and SKY applied simultaneously	Both FISH and SKY	Gene mapping – define amplified genes, monitor a key gene or chromosome region under SKY background	Use fluorescent direct labeled probe for FISH ^a
SKY after immuno-staining	Both immuno-staining and SKY	DNA/protein co-interaction – define specific stages for meiotic prophase, trace the association of a specific protein along or among chromosomes	Use immuno-staining for protein detection

^a The integration of SKY and indirect FISH is feasible. This can be accomplished by the careful selection of the correct combination of antibodies avoiding antibody cross reactivity (involved in both SKY and FISH).

interference during SKY detection, we elected to use SKY detection on previously immunostained slides rather than immunostaining after SKY. Following antibody detection, the immunostained slide was subjected to denaturation, hybridization and detection. It turns out that the SKY procedure created less interference with signals from previous immunostaining. Different from the protocols of FISH-protein co-detection that we have described previously (Heng et al., 1994, 2000), the choice of color for protein co-detection/SKY is a factor for consideration. When comparing the fluorescence of FITC and rhodamine used for antibody detection, our results indicate that it is better to use FITC for immunostaining prior to SKY detection.

The proteins we have tested are the meiotic synaptonemal complex (SC) protein and the centromere protein. Both of these have proved to be successful for combination detection. It is our expectation that this procedure will also work well for proteins that are less abundant than SC proteins. An example of a practical application of this concept is the study of meiotic chromosomes by simultaneously monitoring the synaptonemal complex with co-detection and monitoring the paired chromosomes with SKY during meiotic prophase (Fig. 5). We expect that this particular novel application will have great impact on mouse and human meiotic research (Heng et al., unpublished data).

Direct labeled FISH probe and the reuse of previously FISHed slides

During the experiments of FISH detection on SKYed slides, we observed that direct-labeled probes and indirect-labeled probes responded to the redenaturation procedure differently following a subsequent application of FISH or SKY. We speculate that the difference is due to the involvement of the antigen-antibody interaction that would be accentuated when additional antibody amplification was required for stronger signal detection.

To validate this supposition, we reviewed the results of several repeatedly reused FISH slides, using four pairs of the same probe labeled directly and indirectly. Our results demonstrate that direct-labeled probes are easily removed during redenaturation. By contrast, the indirect labeled and antigen-antibody-amplified probes remain associated with their targets following

redenaturation and caused higher background intensity. Therefore, it is essential that direct-labeled probes should be employed when reutilizing FISH slides. Fig. 6 is an example of the practical applications of this method. Following the fourth application of FISH, the signals of the previous direct-labeled FISH were completely removed and the FISH detection frequency remained over 95%.

Discussion

A comparison of SKY-FISH and immunological co-detection protocols

Applications for SKY-FISH and immunological co-detection protocols are summarized in Table 1. As indicated various combined protocols that were tested can be used advantageously for different applications.

A potentially significant usage of SKY and FISH co-detection is the ability to trace the status of specific genes (like p53) or chromosome regions while monitoring whole genomic changes. This type of integrated analysis could provide important insight by correlating a key gene or region's behavior (such as deletion or amplification) with karyotype instability. For example, in one of the tumor cell lines developed from ATM^{-/-} mice, SKY analysis revealed that specific translocations occurred and involved possible abnormalities of the centromere region. To confirm the centromeric abnormalities involving major satellite sequences, FISH detection was performed using major and minor satellite sequences as a probe on these previously SKYed slides. The FISH and SKY co-detection demonstrated that the abnormal centromeric region is indeed a feature of this tumor line and was not caused by centromeric fusion (data not shown). This combined approach also provides the means to pinpoint the role of specific genes when studying gene amplification.

This experimental system of SKY and protein co-detection was originally developed for use in meiotic research. A representative example was briefly discussed in this communication, however, there are other practical applications that this system can be adapted for use in the functional genomic field. These applications include localizing specific proteins on the chromo-

some/chromatin domain, tracing various stages of the cell cycle with protein markers and comparing the protein binding patterns on chromosomes between wild type and knock out cells to define their function.

The protocol for SKY detection after FISH has been successfully used for prenatal diagnosis. By reexamining previously FISHed slides, we have identified marker chromosomes which were not identified by previous FISH with limited coverage of probes used (data not shown).

The reuse of chromosome slides with fluorescent direct-labeled probes

We have found that it is possible and very practical to apply FISH probes to previously SKYed or FISHed slides. A major challenge that arose was the level of high background; this was accentuated when amplification steps were required during the initial FISH application. Even though indirect FISH was successful for new probe detection, FISH signals of previous loci remained. This may interfere with the detection of the new probes. Our current results with repeated applications of FISH on previously FISHed slides demonstrates that using direct-labeled probes for previous FISH detection without antibody amplification is necessary to reduce the background of subsequent FISH or SKY detection. It is evident that without the antigen-antibody interaction, direct-labeled probes are easily removed during the denaturation process. Signals from old loci following removal will no longer exist and will not interfere with new probes. This has important implications for clinical diagnostics when using multiple sequential FISH probes. This is very practical when different combinations of probes must be tested in single cell FISH analysis of metaphase and interphase preparations. Further evaluation of these protocols for the reuse of previously FISHed slides is under investigation.

Interestingly, our experience with the reuse of SKY and FISHed slides solved one mystery regarding the quality of FISH detection on FISHed slides. By using a biotin-avidin system, rehybridization on previously FISHed slides achieved a measure of limited success. The successful use of FISH was usually associated with a high background (Wang et al., 1995). Sequential FISH procedures using previously FISHed slides can be successful with up to eight rehybridizations, when direct-labeled FISH probes are primarily used (Escudero et al., 1998). Our data show that only direct-labeled probes are easily removed and can generate reliable FISH results when the same slide is reused. There has been no explanation from previous studies as to why biotin-labeled probes are the exception (they cannot be denatured off!). Our comparative studies demonstrate that the success of applying FISH to previously FISHed slides is contingent on avoiding the formation of an antigen-antibody complex. Our experimental results indicate that the reuse of the same slides can be performed 5–10 times if direct-labeled painting probes are used for each rehybridization repetition.

In conclusion, the separate use of SKY, FISH and immunostaining is effective. However, there are situations where combinations of these techniques on the same sample (i.e. slide) would provide the optimal analysis. We have reviewed the plausibility of reusing SKY or FISH slides thereby integrating FISH and SKY detection. These techniques are successful and provide distinct advantages when appropriately applied. The use of combined protein and SKY co-detection is a very effective method able to address questions concerning functional genomics including meiotic research. It is expected that application of this combinatorial approach to the clinical arena will also impact cancer cytogenetics and prenatal diagnosis.

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