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Characterization of all human male synaptonemal complexes by subtelomere multiplex-FISH

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Abstract. During meiotic prophase I, homologous chromosomes synapse and recombine. Both events are of vital importance for the success of meiosis. When homologous chromosomes synapse, a proteinaceous structure called synaptonemal complex (SC) appears along the pairing axis and meiotic recombination takes place. The existence of immunolabeling techniques for SC proteins (SCP1, SCP2 and SCP3) and for DNA mismatch repair proteins present in late recombination nodules (MLH1) allow analyses of both synapsis and meiotic recombination in the gametocyte I. In situ hybridization methods can be applied afterwards because chromatin is preserved during cell fixation for immunoanalysis. The combination of both methodologies allows the analysis of synapsis and the creation of recombination maps for each bivalent. In this work we apply the seven-fluorochrome subtelomere-specific multiplex FISH assay (stM-FISH) to human male meiotic cells previously labeled by immunofluorescence (SCP1, SCP3, MLH1, CENP) to assess its utility for human SC karyotyping. This FISH method consists of microdissected subtelomeric probes labeled combinatorially with seven different fluorochromes. Results prove its usefulness for the identification of all human SCs. Furthermore, by labeling subtelomeric regions this one-single-step method enables the characterization of interstitial and terminal SC fragments and SC delineation even if superposition is present in pachytene spreads.

During meiotic prophase I, when homologous chromosomes pair, synapse and recombine, a proteinaceous structure called synaptonemal complex (SC) forms along the pairing axis. Synapsis and meiotic recombination are of vital importance for the success of meiosis. Synapsis failure or meiotic recombination rate decrease were reported to cause partial or total meiotic arrest (Hultén et al., 1970; Egozcue et al., 2000). Several studies were carried out using silver nitrate or phosphotungstic acid to stain SC for its analysis by light and electron microscopy. These methods were applied indistinctly to gametocytes I of males (Hultén et al., 1974; Navarro et al., 1991) and of females (Garcia et al., 1987). In the last few years the appearance of immunolabeling techniques has renewed the interest for SC. Detection of SC proteins (SCP1, SCP2 and SCP3) and of proteins present in late recombination nodules (MLH1) allow simultaneous analyses of synapsis and meiotic recombination. Due to chromatin preservation during cell fixation, it is possible to combine the immunoassay with FISH techniques allowing the identification of single bivalents (Barlow and Hultén, 1996). Then, synapsis and meiotic recombination patterns can be evaluated for each specific pair of chromosomes (Barlow and Hultén, 1996; Lynn et al., 2002; Tease et al., 2002).

Since 24-color karyotyping techniques were first described (Schröck et al., 1996; Speicher et al., 1996), a great number of different FISH-based multicolor technologies have been devel-

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oped for their application in clinical and cancer cytogenetics (Langer et al., 2004; Liehr et al., 2004). Efforts have been mainly directed to improve the analysis of metaphase chromosomes by increasing both the sensitivity and the number of probes, which can be simultaneously hybridized. Applications of multicolor-FISH approaches to mouse and human SCs were reported. Different DNA probes (chromosome-, locus- or centromere-specific) were used to identify up to four SCs at the same time (Lynn et al., 2002; Tease et al., 2002). The first identification of all SCs was achieved in mouse. For this purpose two rounds of multicolor FISH of chromosome-specific libraries were used (Froenicke et al., 2002). Recently, a multiplex-FISH method using specific centromeric probes (cenM-FISH) was applied to male spermatocytes for the identification of human SCs (Oliver-Bonet et al., 2003) and used for the characterization of human male recombination maps (Sun et al., 2004). The seven-fluorochrome subtelomere-specific FISH assay is another FISH-based strategy developed to improve the detection of subtelomeric rearrangements (Fauth et al., 2001).

In this work, we apply the set of microdissected subtelomeric probes labeled combinatorially with seven different fluorochromes (stM-FISH) to human male meiotic cells previously labeled by immunofluorescence (SCP1, SCP3, MLH1 and centromere proteins) to assess its utility for human SC karyotyping. We demonstrate the feasibility exemplarily on one sample.

Materials and methods

A testicular sample was obtained from a patient undergoing vasectomy under a local anesthetic. Written consent was obtained, and the study was approved by the Institutional Ethics Committee. The testicular tissue was macerated in a hypotonic solution (sodium citrate 1%) and placed in a centrifuge tube to let the seminiferous tubules deposit. The supernatant containing the testicular cells was recovered and centrifuged for 5 min at 600 *g*. Finally, the pellet was resuspended in 1% sodium citrate and diluted to about 3 times. Cell spreading and fixation were performed following a protocol described elsewhere (Barlow and Hultén, 1996) with minor modifications: 10 µl of cell suspension were mixed with 20 µl of 0.003 Photo-Flo solution (Kodak) on a clean microscope slide and allowed to stand for 10 min. Then, 90 µl of 2% formaldehyde-0.02% SDS pH 8.4 were added to the mix. After 10 min, the slides were rinsed in distilled water and allowed to dry at room temperature.

Immunocytofluorescence analysis

Immunolabeling of spermatocytes was performed with slight modifications of the Barlow and Hultén (1998) protocol. Slides were blocked with 0.05 g milk powder in 1 ml 4× SSC-0.05% Tween-20 (4× SSCT) for 30 min. The primary antibodies used were rabbit anti-SCP3 (Lammers et al., 1994) and rabbit anti-SCP1 (Meuwissen et al., 1992) (both gifts from Dr. Christa Heyting; University of Wageningen, The Netherlands), anti-CENP (CREST serum given by Dr. William Earnshaw, University of Edinburgh, UK) and mouse anti-MLH1 (Pharmingen, San Diego, Calif., USA). They were applied at 1:1000, 1:1000, 1:1000 and 1:250, respectively, in blocking mix overnight at room temperature. After three 5-min washes in 4× SSCT, the secondary antibodies, TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG (both from Sigma, Madrid, Spain) were applied at 1:250 for 4 h at room temperature. After 3× 5-min washes in 4× SSCT the rabbit anti-human IgG antibody (Sigma, Madrid, Spain) labeled in Pacific Blue with Zenon Reaction (Molecular Probes, Spain) was applied at 1:250 for 1 h at room temperature. Finally, after washes and a brief rinse in distilled water, slides were air-dried and counterstained with antifade solution (Vector Laboratories Inc., Burlingame, Calif., USA). Evaluation was performed with a fluorescence photomicroscope (Olympus BX60) equipped with a Sensys CCD camera (Photometrics). All observed pachytene nuclei with anti-MLH1 antibody foci were captured and processed using a Power Macintosh G3 with Smartcapture software (Digital Scientific; Cambridge, UK). Slides were stored at –20°C until hybridization.

StM-FISH

For the stM-FISH assay DNA probes were prepared as described (Fauth et al., 2001) with minor modifications. Microdissected subtelomeric probes, sized between 5 and 10 Mb, were amplified and labeled by DOP-PCR according to a combinatorial labeling scheme based on 7 different fluorochromes (Fig. 1, top). For direct labeling DEAC (Perkin Elmer), Cy3 and Cy5 (both from Amersham Pharmacia Biotech), TexasRed (Molecular Probes) dUTP-conjugates were used. DNP (Perkin Elmer), biotin and digoxigenin (both Roche Diagnostics) dUTP-conjugates were used for indirect labeling. After DNA precipitation the probe set was resuspended in the hybridization mix (50% formamide, 20% dextran sulfate and 2× SSC). For the hybridization, antifade solution was removed from SC spreads using 4× SSCT (3 × 5 min at 37°C). After dehydration in 70, 90, 100% ethanol, slides were air dried. Cells were denatured in 70% formamide/2× SSC for 2 min at 69°C, dehydrated by a cold ethanol series (70, 90, 100%) and air dried. Probe solution was denatured for 7 min at 75°C and pre-annealed for 20 min at 37°C. Hybridization was for 48 h at 37°C. After post-hybridization washes, anti-DNP-KLH-Alexa488 (1:400; Molecular Probes), avidin-Cy5.5 (1:200; Rockland Inc.) and anti-digoxigenin-Cy7 (1:50; Cy7 from Amersham Pharmacia Biotech) were used for detection of hapten-labeled probes. Finally, slides were counterstained with DAPI and mounted in p-phenylenediamine dihydrochloride antifade solution (Merck). Visualization was performed using a motorized epifluorescence microscope with an eight-position filter wheel (Leica DMRXA-RF8), a Sensys CCD camera (Photometrics; Kodak KAF 1400 chip) and the Leica QFISH software (Leica Microsystems Imaging Solutions, Cambridge, UK).

The identification of all SCs was performed by projection of the stM-FISH image results into the image of the immunolabeled pachytene cell previously captured.

Results

Pachytene cells of a normal, healthy donor, previously analyzed by immunocytogenetic techniques, were successfully hybridized with stM-FISH. In this approach subtelomere p and q probes of each chromosome are labeled with the same combinatorial fluorochrome pattern. For acrocentrics and chromosome 1 only the q arm is detected. This allowed the characterization of all human male SCs simultaneously with the immunolabeling of SCP1, SCP3, MLH1 and CENP (Fig. 1).

Discussion

The SCP3 protein is part of the SC lateral elements (Schalk et al., 1998). Lateral elements are fully joined at pachytene stage by the central element, where SCP1 is present (Eijpe et al., 2000). On the other hand, the DNA mismatch repair protein MLH1 marks sites where crossing-over has taken place (Barlow and Hultén, 1998). Thus, the combined immunolabeling of SCP1, SCP3, MLH1 and CENP does not only provide information about synapsis of each pair of homologous chromosomes, but also shows the distribution of recombination events and the centromere position in the SC.

As compared to two-color FISH methods applied on SC spreads (Lynn et al., 2002; Tease et al., 2002), multi-color FISH assays allow the identification of the complete SC set of a cell in two (Froenicke et al., 2002) or one single hybridization round

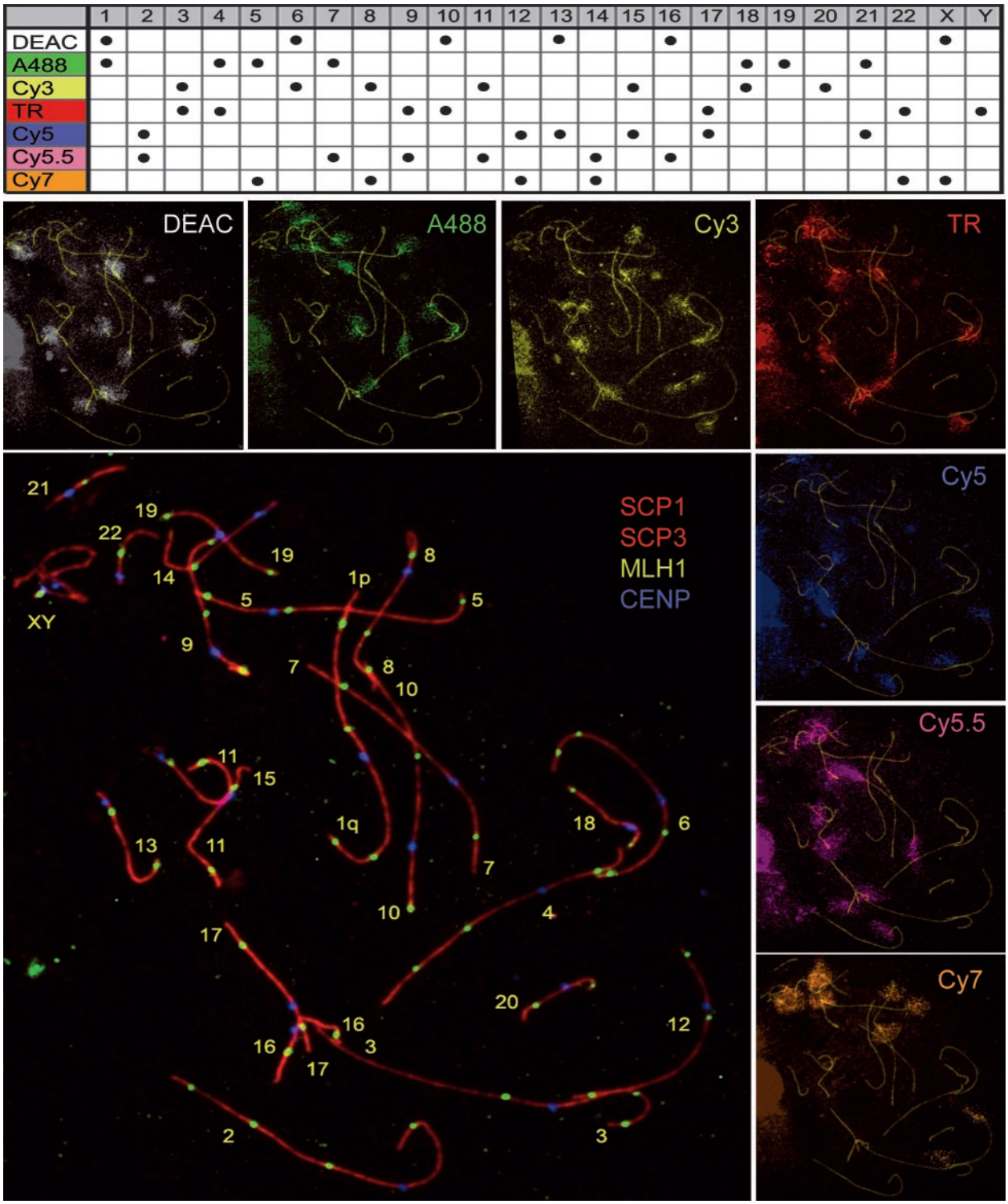

Fig. 1. The table displays the combinatorial labeling scheme for the stM-FISH assay. Small images show stM-FISH results for each fluorochrome superimposed to the corresponding SCP1 and SCP3 cell image. Center image represents an immunolabeled pachytene cell with all synaptonemal complexes identified by stM-FISH (indicated with the corresponding number). SCs are in red (SCP1 and SCP3), MLH1 in green and centromere in blue.

(Oliver-Bonet et al., 2003). As a result, meiotic recombination frequencies and localization, and synapsis can be simultaneously analyzed for each and all SCs of the set. Therefore, abnormal processes in any of the 22 autosomal SCs can be characterized.

The use of a seven-fluorochrome stM-FISH assay has some distinct advantages. For example, pachytene cells sometimes present fragmented or overlapped SCs. In this case, subtelomere probes are even more helpful, because one can follow SCs from one end to the other. The use of seven different fluorochromes allows a two-fluorochrome combination for each chromosome (except chromosome 19, 20 and Y which are labeled with one fluorochrome). This increases both sensitivity and unequivocal classification as has been previously discussed in detail (Azofeifa et al., 2000; Fauth et al., 2001) and facilitates SC identification.

In addition to the quality of hybridization, the correct classification of FISH signals depends on the quality of immunolabeling and spreading. Furthermore, due to the morphology and condensation of chromatin after formaldehyde fixation the hybridization signals appear usually diffuse, which may further hamper the classification. However, the use of seven different fluorochromes results in a reduction of the number of signals per channel, reducing the probability of overlapping signals, thus facilitating a correct signal classification.

In this work, we show for the first time that the complete characterization of all human male SCs by hybridization is feasible using the one-single-step stM-FISH assay (Fig. 1). By using stM-FISH even bivalents with interstitial and terminal fragments in their SC could be identified. At the same time, delineation of superposed SCs has been possible. Moreover, if applied to the meiotic analysis of carriers of balanced chromosome rearrangements, the stM-FISH would exactly determine the position inside the synaptic figure of the chromosomes involved in the reorganization.

In conclusion, results prove that stM-FISH is a reliable method for the identification of all human SC. Furthermore, this one-single-step method enables the characterization of interstitial and terminal SC fragments and SC delineation even if superposition is present in pachytene spreads.

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