

# Interphase fluorescence in situ hybridisation of the X and Y chromosomes in the human eye

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## Abstract

**Aim**—To determine the sex of individual cells in paraffin sections of the human eye by fluorescence in situ hybridisation (FISH) of the X and Y chromosomes.

**Methods**—The authors developed a protocol for FISH of the X and Y chromosomes in paraffin sections of human eyes.

**Results**—In all the specimens that had been fixed in 10% formalin and with a fixation time of up to 3 days sex determination of individual cells was achieved. The percentage of cells with clearly identifiable signals was up to 98% for corneal epithelium, keratocytes, corneal endothelium, trabecular meshwork, lens epithelium, retina, and optic nerve.

**Conclusions**—FISH allows the determination of the sex of single cells in paraffin sections of human eyes without destruction of the tissue structure. Its main application is the histological analysis of sex mismatched corneal, RPE, or neuroretinal transplants to distinguish host and donor cells.

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Some 45 years ago, Pedler and Ashton reported on the presence of the Barr body in tissue sections of the human eye in this journal.<sup>1</sup> However, the presence of the Barr body only gives a statistical probability for the sex of tissue and cannot be used to identify reliably the sex of an individual cell and can only be identified in cell types with large cell nuclei like, for example, epithelial cells.<sup>2</sup>

In recent years, a new technique called fluorescence in situ hybridisation (FISH) has been developed that allows the specific localisation of fragments of DNA in *single* cells by a fluorescence labelled complementary DNA probe which binds to a specific DNA sequence. In this study, we tried to apply FISH of the sex chromosomes on paraffin sections of human eyes.

## Materials and methods

### TISSUE

Twenty eight routine paraffin embedded eyes, either surgically removed or received post mortem, with an equal number of male and female sex and three different fixatives—namely, 10% neutral buffered formalin (20 eyes), 20% neutral buffered formalin (four eyes) or 1% glutaraldehyde, 3% formaldehyde (four eyes) were retrieved. Fixation time for the specimens ranged from 5 hours to 6 weeks. Thin sections of 6 µm were mounted on silanised slides and baked overnight at 65°C. Metaphase spreads

prepared from lymphocytes of a male donor's peripheral blood were used as controls.

### PROBES

We used a digoxigenin labelled X chromosome  $\alpha$  satellite probe (DXZ1, Oncor, Gaithersburg, MD, USA) and a biotin labelled probe for the classic satellite of the human Y chromosome (DYZ1, Oncor).

### FISH PROTOCOL

The slides were deparaffinised with xylene and then hydrated with a series of graded ethanol. Pretreatment with a hot 50% glycerol solution (pH 7.45) at 90°C for 5 minutes, washing in 2× SSC for 2 × 2 minutes and air drying. For digestion, proteinase K solution (10 ml proteinase K (10 mg/ml, from Oncor) in 990 ml 2 × SSC) was applied on the slides which were incubated on a warm plate at 37°C for 45 minutes. Thereafter, they were washed in 2 × SSC for 2 minutes, dehydrated with a series of graded ethanol, and air dried. For denaturation, a 72°C hot formamide solution (5 ml water, 2.5 ml 20 × SSC, 17.5 ml formamide) was applied and the slides were placed on a hot plate at 72°C for 10 minutes. Dehydration was carried out with a series of cold (–20°C) graded ethanol. For denaturation of the DNA probes, the probes were diluted 1:20 in a formamide solution (7 g dextran sulphate, 45 ml formamide, 7 ml 20 × SSC, 18 ml water) and placed into a small plastic tube in a hot water bath at 72°C for exactly 5 minutes. For hybridisation, the denatured probe solution was applied on the slides which were incubated in a humidified chamber at 37°C overnight. The next morning, for post-hybridisation washing the slides were placed into a coplin jar filled with 0.25 × SSC in a waterbath at 72°C for 5 minutes, washed twice in 2 × SSC for 2 minutes each, and air dried. For detection and signal amplification, a dual detection solution containing rhodamine labelled anti-digoxigenin and fluorescein labelled (FITC) avidin (from Oncor) was applied, the slides were covered with a plastic coverslip and put on a warm plate at 37°C for 15 minutes. They were washed in 2 × SSC for 3 × 2 minutes and air dried. Dual amplification was carried out by the sequential addition of goat anti-avidin antibody, fluorescein labelled (FITC) avidin, rabbit anti-sheep antibody, and rhodamine labelled anti-rabbit antibody (all from Oncor). The samples were incubated with each of the latter solutions on a warm plate at 37°C for 15 minutes. They were washed in 2 × SSC for 3 × 2 minutes and air dried. Finally, the slides were counterstained with DAPI (4'-6-diamidino-2-phenyl-indole)/antifade (Oncor) and covered with glass coverslips. All slides were evaluated

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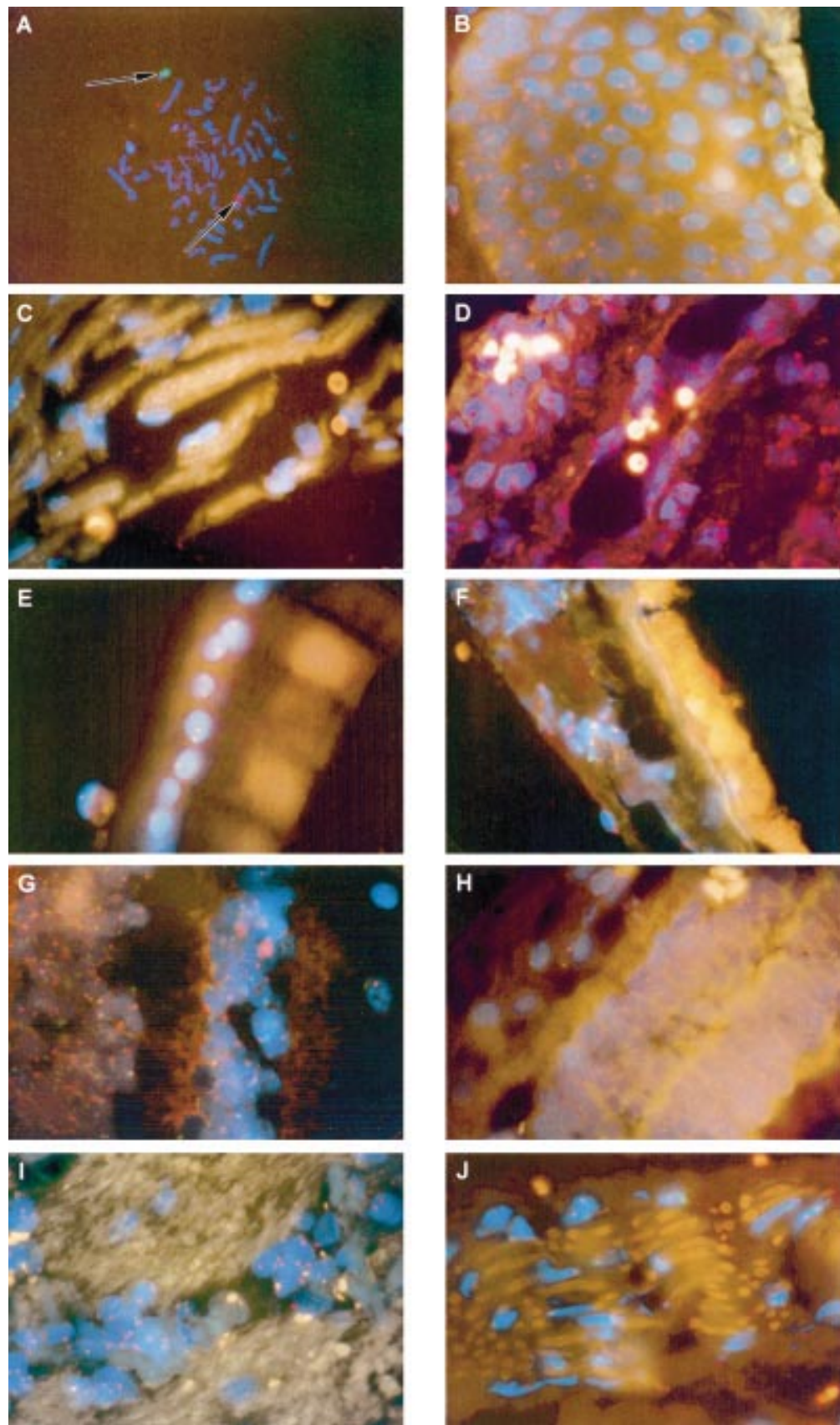


Figure 1 FISH of the the X (red) and Y (green) chromosome in the human eye, counterstained with DAPI. Magnification  $\times 800$ , oil immersion, triple band pass filter. Male cells in (A) control metaphase spread of a male lymphocyte, (C) trabecular meshwork, (F) choroid, (G) retina, and (J) ciliary nerve. Female cells in (B) corneal limbal epithelium, (D) iris, (E) lens epithelium, (H) retina, and (I) optic nerve.

Table 1 Relative frequency of sex chromosome detection for various cell types (16 cases)

Cell type	Percentage of signals
Corneal epithelium	98
Keratocytes	70
Corneal endothelium	80
Iris stroma	85
Trabecular meshwork	85
Non-pigmented ciliary body epithelium	85
Lens epithelium	92
Choroidal cells	80
Retinal pigment epithelium	35
Retinal cells	98
Optic nerve	80
Sclera	60

in oil immersion using a Zeiss axiomatic epifluorescence microscope with a triple band pass filter for the bluish DAPI (365 nm), green FITC (460 nm), and the reddish rhodamine (570 nm). The protocol for the control metaphase spreads of cultured lymphocytes was identical except that they underwent no pretreatment, were not digested, and were denatured for only 2 minutes.

### Results

We were able to develop a uniform protocol allowing the detection of the sex chromosomes for all cell types of the human eye (Table 1). However, only the specimens, both surgically removed and postmortem eyes, that had been fixed in 10% formalin for no more than 3 days displayed signals (16 cases). Tissue fixed for a longer time period, in higher concentrations of formalin, or in glutaraldehyde could not be successfully analysed.

The size of the cell nuclei was increased by about 0.5 as a result of the glycerol pretreatment. The detected signals for the X chromosome appeared like red dots, the Y chromosome appeared as a more diffuse green dot (Fig 1A). Two red signals were visible in female cells (Fig 1B, D, E, H, I) and a green and a red signal in male cells (Fig 1A, C, F, G, J). Both signals were located within the nuclei of the cells. The signals were often at different levels of focusing so that the slides had to be checked routinely at various focusing planes. The percentage of cells with clearly identifiable signals was high for most cell types (Table 1). The signals were still detectable after 1 year. The oldest specimen that was analysed successfully was 30 years old. An intense orange yellowish autofluorescence was present in the numerous lipofuscin granules of the RPE cells in adult eyes (Fig 1F).

### Discussion

For the first time, the dual FISH technique for the simultaneous detection of the two sex chromosomes was applied successfully to routine paraffin sections of human eyes. It was possible to develop a uniform hybridisation protocol which allowed the simultaneous analysis of the sex chromosomes in individual cells of all the different cell types of the human eye.

FISH was not possible in eyes previously fixed in glutaraldehyde or in formalin concentrations higher than 10% and after a fixation time longer than 3 days. This finding can be explained by the fact that fixation of tissue results in extensive cross links between nuclear proteins and DNA as well as in depurination and degradation of DNA.<sup>3</sup> But, in the eyes fixed with 10% formalin for up to 3 days, hybridisation was possible only after pretreatment with hot glycerol solution and 45 minutes of digestion with proteinase K, which makes the DNA strands better accessible for the probes.<sup>4</sup>

Fewer signals were found in the spindle-shaped keratocytes and scleral cells because of nuclear slicing in the sections as a result of the anisotropic shape of their nuclei. Signal counts in the adult RPE cells were lower because they were often concealed by autofluorescent lipofuscin granules.<sup>5</sup>

The main advantages of the FISH method over Barr body staining are, firstly, the possibility of determining the sex of an individual cell.<sup>1</sup> Secondly, the sex chromosomes can be detected in all cell types of the eye whereas Barr bodies have only been found in corneal epithelium, iris stroma cells, ciliary body epithelium, stromal cells of the ciliary body, lens epithelial cells,<sup>6</sup> and in the ganglion and nuclear cells of the retina.<sup>1</sup>

A disadvantage of the FISH technique is that the probes that were used are specific for human sex chromosomes and can therefore not be used for animal studies as has been possible with the sex chromatin method.<sup>7</sup>

A minor future application of the FISH technique might be the identification of mislabelled cases in the pathology laboratory if they come from people of different sex.<sup>8</sup> However, the most interesting ophthalmic application is the analysis of sex mismatched transplants in which the sex chromosomes can be used as a cellular marker to distinguish host and donor tissue. By this means, we were able to analyse successfully the survival of cells in corneal transplants.<sup>9</sup> Of course, the same technique can be also used for the analysis of transplants of corneal stem cells, retinal pigment epithelium,<sup>10</sup> or neuroretinal transplants as it has also been shown for other organ transplants like heart,<sup>11</sup> liver, bone marrow,<sup>12</sup> or lung.

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