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Spreading and staining of human metaphase chromosomes on aminoalkylsilane-treated glass slides

A. C. VAN PROOIJEN-KNEGT, A. K. RAAP, M. J. M. VAN DER BURG, J. VROLIJK and M. VAN DER PLOEG

Department of Histochemistry and Cytochemistry, Sylvius Laboratories, Wassenaarseweg 72, NL-2333 AL Leiden, The Netherlands

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Summary

The properties of aminoalkylsilane-treated glass slides for the preparation of metaphase spreads and their staining quality have been studied and compared with those of slides which had only been cleaned in ethanol/ether. The parameters investigated were: (1) the average area of metaphases from cultures of blood from both healthy donors and haematology patients; (2) the influence of the positively charged 'coating' on the quality of quinacrine- and Giemsa-banding patterns; (3) non-specific background staining for these banding methods; (4) the number of metaphases as compared to the number of interphase cell nuclei per area of preparation; and (5) the Feulgen-staining intensities of chromosomes and chicken erythrocyte nuclei.

The quality of metaphase preparations and the differential staining of chromosomes is better on aminoalkysilane-treated glass slides than that of preparations on routinely cleaned normal microscope slides. In the preparations on aminoalkylsilane-treated slides, the distribution of the cells over the glass surface is more homogeneous; and no influence could be detected on the relative frequency of metaphases as compared to the number of non-divided cell nuclei; the average area per metaphase is increased by about 10% and consequently the number of overlapping chromosomes is decreased.

Preparations on aminoalkylsilane-treated glass, after Q-, G- and DAPI-banding procedures, always showed less binding of the staining compounds to the glass slide (a cleaner background) than those on routinely cleaned microscope glass slides. The Feulgen-pararosaniline staining intensities of human metaphase chromosomes and chicken erythrocyte nuclei are the same on aminoalkylsilane-treated slides and on routinely cleaned glass slides. Furthermore, the reproducibility and constancy of quinacrine banding was improved by development of an equilibrium staining method which does not require a washing procedure. The medium, containing 0.002% quinacrine, allows optimal staining results to be obtained for microphotography purposes within 30 min of staining (for visual inspection at least 90 min is required) and is used as the embedding medium.

In combination with aminoalkylsilane-treated glass slides, this procedure leads to a clean background and reproducible banding patterns of excellent quality, the results being better and more constant than those of methods described before.

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Introduction

The purpose of the present study was to improve the quality and the reproducibility of banding procedures for human metaphase chromosomes. Special attention was paid to two parameters: non-specific background staining in the microscope slides, and the heterogeneous staining which readily occurs during the washing procedure after quinacrine staining.

The background staining that occurs when compounds like quinacrine, quinacrine mustard, DAPI and Giemsa are applied for the differential staining of metaphase chromosome preparations is the result of a strong binding capacity of these dyes to glass. This binding could be caused by the electrostatic attraction between the negative silicate groups of glass and the positive groups of the basic dye compounds and, based on this hypothesis, it was expected that coating the glass surface with positive groups, such as amino groups would diminish this type of background staining.

To overcome the problem of background staining, glass slides were treated with 3-aminopropyltriethoxysilane which provides the glass surface with alkylamine groups. It was found that aminoalkylsilane-treated slides, in accordance with expectations, showed hardly any background staining after quinacrine or Giemsa incubation. Two favourable side effects of the aminoalkylsilane treatment were observed: better spreading of the metaphase plates and a more homogeneous distribution of the cells over the slide.

Heterogeneity in quinacrine staining results readily arises during the differentiation procedure necessary in quinacrine staining methods published so far. Rinsing is critical, and has to be done briefly in order to prevent the removal of all quinacrine, and this may lead, when not performed carefully, to preparations in which parts are still overstained while other parts have already lost too much of the dye to show a clear banding pattern.

To solve the problem of heterogeneous staining intensities among the metaphases, a quinacrine staining procedure was developed in which the concentration of the dye compound is so low that it is not necessary to wash the excess of quinacrine away after staining. Due to the lower dye concentration in the staining medium, longer staining times are necessary, but equilibrium-stained preparations embedded in the staining solution clearly show a more constant high quality banding pattern all over the preparation.

Materials and methods

AMINOALKYLSILANE-TREATED GLASS SLIDES

Following the procedure described by Robinson *et al.* (1971), microscope glass slides were cleaned by incubation overnight in a 10% solution of Extran MA01 (alkalisch, E. Merck, Darmstadt, Germany) in distilled water, rinsing with hot (60° C) tap water and with distilled water and drying at 80° C. These slides were then incubated for 16 h in a 2% solution of 3-aminopropyltriethoxysilane (Aldrich Europe, Beerse, Belgium) in dry acetone. Thereafter, the slides were rinsed in acetone and two changes of distilled water and stored in 0.02% NaN_3 in

distilled water. Immediately before use, the slides were rinsed in distilled water and air dried. Slides could be stored for up to six weeks without losing their favourable properties such as the reduced binding of basic dye compounds. Results obtained with slides which had been stored dry at room temperature were not significantly different when these slides were used within two weeks after preparation.

CHROMOSOME PREPARATIONS

Metaphase preparations on microscope slides were prepared from human total blood cultures as described in detail by Bosman *et al.* (1975). By this method chromosome preparations on glass are obtained which show a relatively low protein background and a good metaphase spreading. After the hypotonic treatment and centrifugation, the resulting cell pellet is resuspended in the tube with the last drops of supernatant. About 5 ml of freshly prepared methanol/acetic acid fixative is then gently mixed with the cell suspension by slowly aspirating the contents of the tube into a Pasteur pipette already filled with the fixative. The erythrocyte remnants then dissolve and repeated replacement of the fixative removes non cellular-bound proteins. The blood samples were obtained from healthy volunteers as well as from haematology patients.

STAINING PROCEDURES

Quinacrine staining was performed in two ways: (1) Slides were incubated for 5 min in McIlvaine buffer, pH 4.1, and stained for different periods varying from 10 min to 4 h in an excess of 0.002% quinacrine (G. T. Gurr–Searle, High Wycombe, Bucks, U.K.) dissolved in the buffer. These preparations were mounted in the staining medium itself, and sealed with Fluoromount (Gurr). (2) After pre-incubation in McIlvaine buffer, pH 4.1, for 5 min and staining of the preparations in a 0.1% quinacrine solution in the buffer during 5 min, the preparations were washed with two short buffer rinses and mounted in the McIlvaine buffer.

DAPI staining

Chromosome preparations were stained in a 0.3 μg/ml solution of DAPI (Serva, Heidelberg, Germany) in McIlvaine buffer, pH 7.0, for 20 min. Slides were washed with distilled water and McIlvaine buffer, pH 5.5, for 10 s and embedded in the same buffer.

Giemsa banding

Metaphase preparations were stained following the method described by Sumner *et al.* (1971) with minor modifications. Slides were incubated for 1 h at 65° C in 2xSSC (0.3 M NaCl, 0.03 M trisodium citrate, pH 7.0) after which they were rinsed in running demineralized water. Then the slides were stained for 15 min in freshly made Giemsa medium [1 part of Giemsa solution (Gurr's Giemsa R66) and 19 parts of Gurr buffer pH 6.8 (prepared with buffer tablets) filtered through prefolded filter paper], washed in running demineralized water, and air dried.

Feulgen staining

After fixation in a freshly prepared mixture of methanol, formaldehyde solution 35% (w/v) and glacial acetic acid (85 : 10 : 5, by volume) for 1 h at room temperature, chromosome preparations were stained with the pararosaniline(SO_2) reagent described by Duijndam & Van Duijn (1973). The Schiff reagent was prepared according to Graumann (1952–1953) from pararosaniline ('Acridinfrei', Chroma, Stuttgart, Germany). The stained chromosome preparations were passed through an ethanol–xylene dehydration series and were mounted in a mixture of Fluoromount and Cargille oil as described by Van der Ploeg *et al.* (1977).

FLUORESCENCE MICROSCOPY, MICROPHOTOGRAPHY AND SCANNING CYTOPHOTOMETRY

Chromosome preparations were examined with a DIALUX 20 microscope (Leitz GmbH, Wetzlar, Germany) provided with epi-illumination and a mercury arc (HBO 200 W, Osram GmbH, Berlin, Germany). Visualization of quinacrine fluorescence emission was obtained with a combination of a Calflex heat absorption filter (Balzers AG, Lichtenstein) and a band interference filter AL 436 (Leitz) in the excitation light path; a dichroic mirror (λH 455); and a barrier filter KP 490 (Leitz).

DAPI fluorescence emission was observed with the same microscopic set-up, using a UG 1 (Schott & Gen., Mainz, Germany) and a BG 38 (4 mm, Leitz) combination in the excitation beam; a dichroic mirror (λH 400, Leitz) and an LP 460 (Schott) as a barrier filter.

A Neofluar 100×/1.30 objective (Carl Zeiss, Oberkochen, Germany) was used both for visual inspection and for fluorescence photography on 35 mm Kodak High Contrast Copy Film (Eastman Kodak Comp., Rochester, New York, U.S.A.)* after pre-exposure of the film emulsion as described by Van der Ploeg *et al.* (1976). The actual exposure time for chromosomes stained with quinacrine was 10 s. Photography of DAPI fluorescence emission took 5 s.

Absorption photography of the Feulgen- or Giemsa-stained preparations was performed as described previously (Van der Ploeg *et al.*, 1974a,b) with an AL 559 interference filter (Schott) inserted in the illumination beam. Agfa-Ortho 25 professional (Agfa-Gevaert, Antwerp, Belgium) was used as photographic emulsion. The exposure times were chosen so that the optical densities of the negatives were located on the straight part of the Hurter and Driffield curve of the film emulsion.

For the scanning procedure, the negatives were embedded in immersion oil between glass slides and sealed with Fluoromount. Interactive scanning of individual No. 2 chromosome images was performed using the HIDACSYS-PROFILSCAN or -ARRAYSCAN program (Bosman *et al.*, 1977; Van der Ploeg *et al.*, 1977). The stage-scanner was a CYTOSCAN SMP (Carl Zeiss) interfaced to a PDP 11/04 computer (Digital Equipment, Maynard, Massachusetts, U.S.A.). Sample and line separation were 40 μm in the micrographs (which equals a spatial resolution of 0.125 μm at the original specimen plane); light transmittance values were quantized in 512 linear intensity levels, which were then converted into absorbance values.

Scanning absorbance measurements of Feulgen-stained chicken erythrocyte nuclei and metaphases were performed directly in the preparations with the SMP stage scanner at 560 nm, using the FASTSCAN program. Sample and line separation were 0.25 or 0.50 μm; further details were as specified for the photographic negative scanning.

Determination of the quality of metaphase spreading was performed directly in the Giemsa-stained chromosome preparations with a TAS television scanner (Leitz). For this purpose, a computer program was used that differentiated between metaphases, artefacts and nuclei, and determined the area of each recognized metaphase in pixels together with the number of metaphases and nuclei detected in a scanned area. Determination of the area of a metaphase is performed by dilating (Serra, 1974) the areas of its individual chromosomes until finally one object is obtained (Fig. 1a), which then is eroded as many times as originally the chromosomes had been expanded (Fig. 1b). In each preparation three areas of 10 × 10 microscopic fields (each 0.105 mm^2) were scanned. The areas were taken centrally, 1 cm apart along the long axis of the glass slide.

*Because the High Contrast Copy Film is no longer available, we switched recently to Kodak Technical Pan Film (ESTAR-AH Base) SO-115 with D 19 as the developer. The sensitivity of this film emulsion is higher (the actual exposure time for both types of quinacrine-stained preparations is 2 s, for DAPI fluorescence 0.5 s), and the grain size is sufficiently fine.

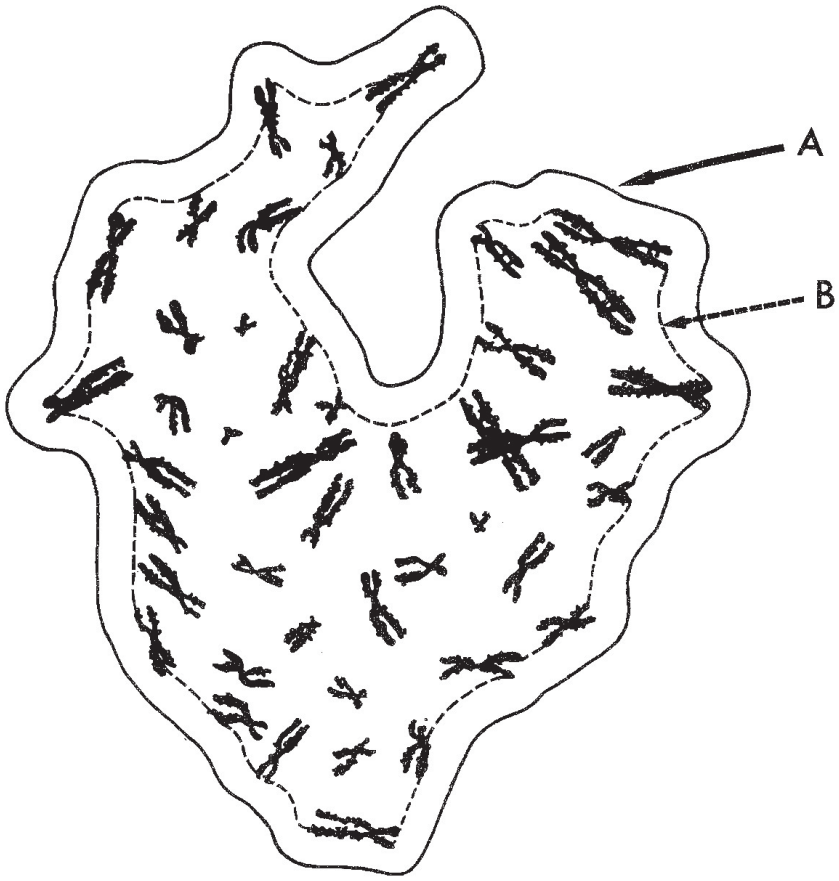

Fig. 1. The determination of the area of a metaphase. (A) Schematic representation of the image resulting after dilation of the thresholded individual chromosomes of a metaphase until one object is obtained. (B) The area of the metaphase, which results after erosion of image (A) as many times as the individual chromosomes had been dilated.

Results

The quality of quinacrine staining results

Visual evaluation was performed 'blind' by five cytogeneticists independently on the preparations themselves, and also on photographic prints of metaphases which had been spread on:

(A) aminoalkylsilane-treated glass slides or;
(B) microscope glass slides cleaned with ethanol/ether, after staining with quinacrine, using either (I) the 'routine' method (0.1%, 5 min, washing), or (II) the equilibrium method (0.002%, 2 h).

The results led to the following conclusions:

(1) preparations on 3-aminopropyltriethoxysilane-treated glass *always* showed less background (glass) staining than those on alcohol/ether-cleaned slides.

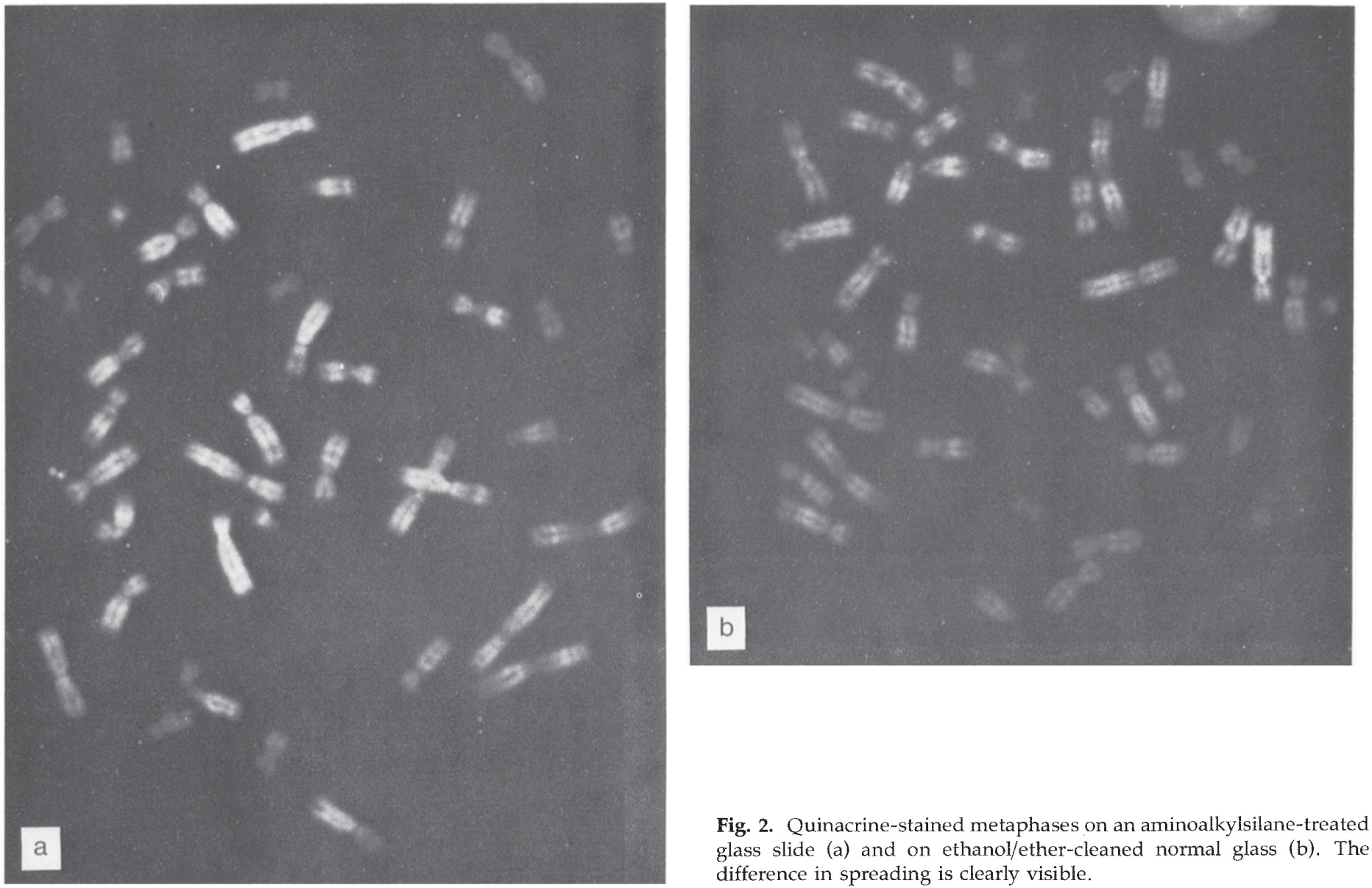

Fig. 2. Quinacrine-stained metaphases on an aminoalkylsilane-treated glass slide (a) and on ethanol/ether-cleaned normal glass (b). The difference in spreading is clearly visible.

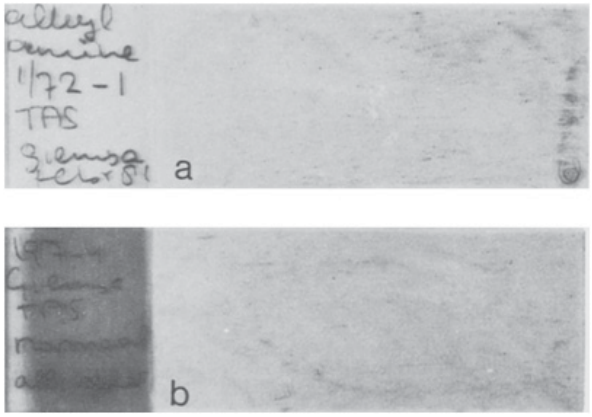

Fig. 3. Giemsa-stained metaphase preparations on aminoalkylsilane-treated glass (a) and on alcohol/ether-cleaned slides (b). The decreased background staining in the first type of preparation can already be observed macroscopically, especially in the ground end of the slide.

(2) the results after routine staining sometimes showed variations in quality as expected because of the washing procedure. The overall quality of the banding pattern(s) of the equilibrium-stained preparations was more uniform and always better than that in the routinely stained preparations.
(3) the distribution of cells and metaphases over the slides is more homogeneous than in glass slides cleaned with ethanol/ether.
(4) the chromosomes of metaphase spreads on aminoalkylsilane-treated glass slides seemed to be spread over larger areas than those on ethanol/ether-cleaned glass (Fig. 2).

Giemsa-banded metaphases from both types of preparations were similarly evaluated visually. In all aminoalkylsilane-treated glass preparations, the overall background staining intensity is lower, as can already be observed macroscopically (Fig. 3). The quality of the banding patterns was good in both the routinely cleaned and the aminoalkylsilane-treated glass preparations, and no significant difference could be detected.

The results of DAPI staining showed no evident difference either, except that in the aminoalkylsilane-treated glass preparations the background is also cleaner than that in ethanol/ether-cleaned slides.

Metaphase spreading

The spreading of metaphases prepared from blood cell cultures of three healthy donors and three haematology patients (one bone marrow and two lymphocyte cultures) was measured and compared for preparations on aminoalkylsilane-treated glass slides and glass slides cleaned with ether/ethanol. The results are shown in Table 1. Except for the metaphases of the bone marrow culture (D), the mean metaphase areas on all

Table 1. Average areas of Giemsa-stained metaphases (expressed in pixel points ± S.E.M.) determined with a TAS in three areas of 10.5 mm^2 in each of four preparations on aminoalkylsilane-treated glass slides and on routinely cleaned slides.

		Aminoalkylsilane-treated glass	*Alcohol/ether cleaned glass*
Healthy donors	A	639 (±12.1)	564 (±3.9)
	B	629 (±3.8)	581 (±1.7)
	C	689 (±11.7)	581 (±9.9)
Patients	D*	395 (±5.9)	440 (±6.6)
	E	702 (±16.8)	554 (±13.8)
	F	631 (±15.8)	538 (±11.8)

*Bone marrow culture.

aminoalkylsilane-treated glass preparations were larger than those on normal glass slides. Less cells were counted in the central parts of the aminoalkylsilane-treated glass preparations than in routine preparations, but microscopical inspection showed that in the latter preparations, the cells had spread more homogeneously all over the glass slides. The ratio between the number of metaphases and the number of nuclei per area was found to be the same for both types of preparations.

Quinacrine staining intensity

Visual comparison of the staining results obtained with the equilibrium method, using the low concentration of quinacrine (0.002%), after different staining times, showed that sufficient contrast and banding for visual observation was obtained after about 90 min. for the experiments described hereafter, equilibrium staining was always performed for 120 min. The fluorescence emission intensity of the equilibrium-stained preparations seemed visually to be slightly less than that of optimally stained 'routine' preparations. The staining intensities of routinely and equilibrium-stained preparations were then compared by determining the exposure times necessary to obtain fluorescence microphotographic negatives with the same optical densities. The results of this experiment show that equilibrium-stained preparations have at least the same emission intensity as the preparations stained with the 0.1% medium. Under the illumination conditions applied, using the High Contrast Copy Film these exposure times were 10 s for brightly fluorescent metaphases in preparations stained with the 0.1% quinacrine medium, and 6–7 s for equilibrium-stained (0.002%) metaphases. With the more sensitive Technical Pan Film, a 2–5 s actual illumination time is necessary to obtain microphotographs with sufficient contrast (Fig. 4).

Feulgen–DNA absorbance values

To check a possible influence of the aminoalkylsilane coating on the Feulgen stainability, the absorbance values of chicken erythrocyte nuclei stained in preparations on both types of glass slides were measured. The mean integrated A_{560} per 0.25 μm^2 of 60 nuclei (ten in each of three areas in two slides) per type of preparation were measured.

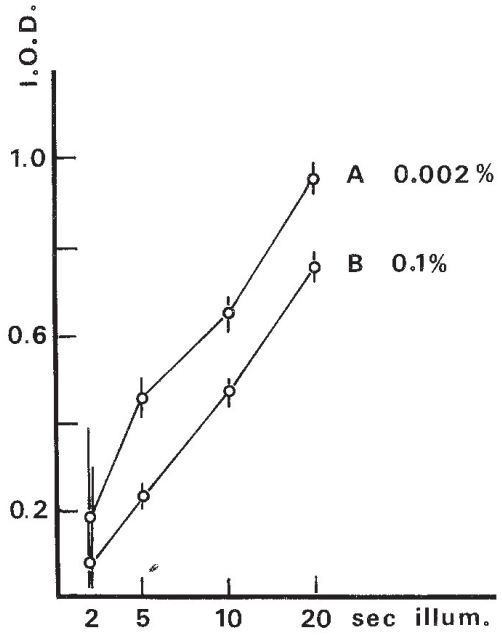

Fig. 4. The relations between integrated corrected optical density values in microfluorographic negatives on Technical Pan Film of quinacrine-stained chromosomes no. 2, and the actual exposure time. Each point is the mean of six measurements (vertical bars = S.D.).

The means ±S.E.M. were:

alcohol/ether-cleaned glass : 26.45 ± 0.13
aminoalkylsilane-treated glass: 26.41 ± 0.15.

The mean Feulgen–DNA absorbance (±S.E.M.) of human no. 2 chromosomes measured as the optical density per 1/64 μm^2 in photographic negatives were for preparations on:

alcohol/ether-cleaned glass : 40.79 ± 0.45
aminoalkylsilane-treated glass: 40.77 ± 0.45.

To check whether the difference in staining procedure influenced the intensity of Feulgen staining (carried out after quinacrine staining), the integrated Feulgen absorbances were determined for no. 2 chromosomes from photographic negatives. The results are shown in Table 2.

Table 2. Integrated optical density values of human metaphase chromosomes no. 2 after Feulgen–pararosaniline staining measured on photographic negatives. Each value represents the mean of 20 chromosomes (10 in each of two preparations).

Preceding treatment	*Integrated optical density per 1/64 μm^2* (±S.E.M.)
A —	408 (4.5)
B quinacrine 0.1%	414 (4.5)
C quinacrine 0.002%	409 (14.3)

Discussion

Treatment of porous glass beads with aminoalkylsilane, followed by chemical conversions, has enabled RNA and heat-denatured DNA to be attached for affinity chromatographic purification of nucleic acid binding proteins (Robinson *et al.*, 1971). In the present study, glass slides were treated with 3-aminopropyltriethoxysilane to reduce or prevent non-specific binding of basic dyes to the glass surface. This principle worked in practice as can be concluded from the reported results. Aminoalkylsilane-modified glass slides also were found to decrease the non-specific background staining when treated with a fluorochrome-labelled antiserum (Van Prooijen *et al.*, unpublished results). An unexpected effect of the aminoalkylsilane treatment of glass which was observed, is that metaphases spread better on such slides than on ethanol/ether-cleaned glass slides.

As a result of the special culture and preparation procedure applied in our laboratory, metaphase chromosomes have already been relatively well-spread on normal glass slides (Fig. 2b). However, the average area of metaphases in preparations on aminoalkylsilane-treated glass is 11% larger. In agreement with this larger area, fewer overlapping chromosomes are found in these metaphases. In our preparations the average number of overlaps per metaphase is two per metaphase spread on routinely cleaned glass slides and one on aminoalkylsilane-treated glass slides. Furthermore, the distribution of the cells over the aminoalkylsilane-treated glass slides is more homogeneous over the total area than in preparations on normal glass where they sometimes tend to remain closely around the position where the drop with cell suspension hit the microscope slide.

The reproducibility and the constancy of the quinacrine staining results were further improved by the development of a staining method that does not require a washing procedure to remove any excess of dye. A medium containing a very low concentration (0.002%) of quinacrine allows optimal staining results to be obtained within 90 min and can also be used as an embedding medium. In this way slides were prepared showing high-quality banding patterns in all well-spread metaphases. This equilibrium staining method works both on routinely cleaned and on aminoalkylsilane-treated glass slide preparations. The staining intensity obtained in these preparations after 30 min visually seemed to be slightly less than in the best-stained metaphases when using the 0.1% medium. This phenomenon most probably can be ascribed to the (weakly) fluorescent background resulting from the quinacrine-containing embedding medium. The exposure times necessary to produce microphotographic negatives of a defined contrast are shorter for equilibrium-stained preparations (120 min) than for preparations stained with the 0.1% medium (Fig. 4).

Neither the aminoalkylsilane treatment of the glass slides nor the equilibrium staining influence the photodecomposition of DNA which occurs during the observation of quinacrine-stained chromosome preparations in the fluorescence microscope. During observation or microphotography of quinacrine-stained chromosomes preparations (for reasons of pre-identification) a decrease of Feulgen stainability can result, as was

described by Bosman *et al.* (1977). It can be concluded from their results that this decrease is related to the strength and duration of irradiation and to the amount of stain bound to the chromosomes.

Our results also show that the decrease in Feulgen stainability for both types of glass and independent of the staining procedure is, at least partially, correlated with the brightness of the fluorescence emission. During prolonged irradiation, the Q-band regions lose more Feulgen stainability than the interbands, which can result in a detectable 'reverse Feulgen-banding' pattern.

In conclusion, the use of aminoalkylsilane-treated microscope glass slides has several advantages. Their preparation is relatively easy; spreading of metaphases on the glass surface is improved, leading to less overlap of chromosomes; non-specific background staining is decreased not only for quinacrine-staining, but also for Giemsa and DAPI staining. Another step towards a more consistent production of high quality chromosome banding has been made by the development of an equilibrium quinacrine-staining method. Both procedures contribute to the quality and reproducibility of chromosome staining results and in this way to a more optimal visual or automated chromosome analysis.

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