CELL-TO-SUBSTRATE CONTACTS IN LIVING FIBROBLASTS: AN INTERFERENCE REFLEXION STUDY WITH AN EVALUATION OF THE TECHNIQUE

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SUMMARY
The closeness of contact between cultured chick heart fibroblasts and glass substrates has been examined by interference reflexion microscopy. Evaluation of the optical technique demonstrated that both the film of medium between the cell and substrate and the cell itself act as two superimposed thin films which generate a complex interference pattern in reflected light at low illuminating numerical aperture (I.N.A.). The interference pattern generated by the cell consists of first and higher orders of interference and can be eliminated from the image by increasing the I.N.A. to > 1.0. The residual zero-order interference pattern at high I.N.A. originates from the thin film of medium between cell and substrate and corresponds to the closeness of contact between cell and substrate. Based on the zero-order interference pattern and a comparison of the same cells with differential interference optics, the following features were recognized in living chick heart fibroblasts. Focal contacts, 0.25-0.5 μm wide, 2-10 μm long, separated by 10-15 nm from the substrate, are located under the peripheral regions of the leading lamellae and near the edge of extended non-spreading regions of the cell margin in moving and stationary cells. Individual focal contacts are coincident with cytoplasmic fibres of the same dimensions as the contact or with the peripheral ends of longer fibres that extend from the focal contact toward the centre of the cell. In spreading cells a second type of contact is present under the peripheral regions of the leading lamellae. This close contact consists of a broad area of uniform cell-to-substrate separation distance (ca. 30 nm). Focal contacts are distributed within the close contact under the leading lamellae. Lamellipodia extend ahead of the close contact and are separated by 100 nm or more from the substrate. Depending on the previous motile history of the cell, the close contact extends to varying degrees under the centre of the cell but it is typically dissected here by discrete areas of distinctly greater cell-to-substrate separation (100-140 nm). Evidence for the adhesive nature of the focal contacts is considered. The common identity is discussed of the focal contacts and associated cytoplasmic fibres described here in living cells with the regions of closest apposition to the substrate and associated cytoplasmic plaques and bundles of microfilaments seen in EM studies.

INTRODUCTION
Movement of fibroblasts on planar substrates is characterized by the advance or spreading of local regions of the cell margin which tends to attenuate the cell, and by the intermittent, abrupt, snapping forward of extended cell processes (Algard, 1953; Trinkaus, Betchaku & Krulikowski, 1971). Spread fibroblasts, therefore, must either be under tension or develop tension transiently. Microdissection studies performed

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on a variety of living cell-types demonstrate that mechanically stable cell-to-substrate contacts capable of withstanding tension occur beneath spreading portions of the cell periphery (Goodrich, 1924; Chambers & Fell, 1931; Algard, 1953; Harris, 1973). However, Harris himself points out the difficulty with this method of distinguishing many closely spaced contacts from single broad adhesions.

The cytoplasm in the vicinity of spreading activity most often assumes the form of a flattened lamellar zone which has been termed the leading lamella (Ingram, 1969; Abercrombie, Heaysman & Pegrum, 1970). Discrete structures circumstantially related to cell-to-substrate adhesion have been described in the peripheral regions of the leading lamella from electron-microscopical studies. These plaques, or condensations of amorphous material and microfilaments subadjacent to the plasma membrane, occur at the points of closest cell-to-substrate contact (Abercrombie, Heaysman & Pegrum, 1971; Brunk, Ericsson, Pontén & Westermark, 1971). The microfilaments in the plaques appear to be continuous with linear arrays of microfilaments passing posteriorly into the cytoplasm in chick heart fibroblasts (Abercrombie et al. 1971). Similar linear arrays of microfilaments are present in a wide variety of fibroblasts and have been demonstrated in many cases to exhibit actin-like properties (Spooner, Ash, Wrenn, Frater & Wessells, 1973; Ludueña & Wessells, 1973; Goldman & Knipe, 1973; Lazarides & Weber, 1974) and to be associated with myosin-like (Weber & Groeschel-Stewart, 1974) and tropomyosin-like (Lazarides, 1975) proteins. The presence of these contractile proteins has led several workers to postulate that the linear arrays of microfilaments are responsible for generating the forces required to move the trailing cell body (Abercrombie et al. 1971; Wessells, Spooner & Ludueña, 1973; Ludueña & Wessells, 1973; Huxley, 1973).

Therefore it is important to examine in living fibroblasts the distribution and formation of the closest cell-to-substrate contacts, their relationship to internal cytoplasmic structures, and hence potential role in fibroblast movement. It is equally important to determine whether these discrete structures represent the only type of cell-to-substrate contact involved in fibroblast motility, or whether broader contacts are involved, especially in the local advances of the cell margin.

We have attempted to answer questions concerning the nature and advance of cell-to-substrate contacts by utilizing the technique of interference reflexion microscopy. Devised by Curtis (1964), the method treats the layer of medium between the cell and coverglass as a thin film capable of generating an interference pattern in reflected light. The colours produced in white light or the intensities in monochromatic light are related to the optical thickness of the film, and therefore provide information on the closeness of contact between the cell and substrate. In our hands the method has demonstrated the existence of 2 distinct types of contact between chick heart fibroblasts and glass substrates: (1) a broad zone of uniformly close contact under the leading lamella of moving cells; and (2) discrete areas of very close contact, or focal contacts, lying under the peripheral regions of leading lamellae or other extended processes. However, in setting up the equipment for interference reflexion microscopy we found that the interference image, especially at the low illuminating numerical aperture used by Curtis, was more complex than would be expected if it were generated
**Cell-to-substrate contacts in fibroblasts**

solely by the thin film of medium between cell and substrate. In evaluating the image we found that the cell itself acts as a second thin film and contributes to the total interference reflexion image. In this paper we present our evaluation of the image, a method for preferentially selecting the contribution to the image of the thin film of medium, and an account of the types and distribution of contacts between the cell and its substrate. An analysis of the formation of contacts during cell movement is presented in a subsequent paper (Lochner & Izzard, in preparation). A preliminary report of both aspects of the work appeared elsewhere (Lochner & Izzard, 1973).

**METHODS**

**Cell cultures**

Primary explant cultures of chick heart fibroblasts were prepared from fragments of 7-day heart ventricles. The fragments were cut in Puck's saline, washed in culture medium and transferred to small drops of culture medium on sterile coverglass substrates (see below). The latter were sealed to sterile Fisher-Littman well slides with Valap (Vaseline, lanolin, paraffin 1:1:1), inverted, and incubated overnight at 37 °C to allow attachment and outgrowth of the cells. Cultures were used between 18 and 24 h of incubation. The culture medium consisted of Basal Medium (Eagle) in Hanks' salts (pH 7.4) supplemented with 10 % horse serum, 10 % embryo extract and 50 U./ml penicillin and 50 μg/ml streptomycin. All components were supplied by GIBCO, Grand Island, N.Y., except for embryo extract which was prepared locally at 3-6 week intervals.

Two types of preparation were used for microscopic observation. When it was necessary to examine the cells sequentially by the interference reflexion method and transmitted-light techniques, the coverglass carrying the culture was supported on spacers in an inverted position over a 35 x 50 mm coverglass bearing a pool of fresh medium. The preparation was sealed with Valap. For high-contrast interference reflexion images and especially for the evaluation of interference colours, the cultures were inverted over a 20 x 20 x 6 mm deep, medium-filled, chamber cast in a flat block of black Silastic (Dow Corning, Midland, Mi.). This procedure minimized extraneous background reflexions. Both preparations were maintained at 37 °C with a Sage air curtain incubator.

**Substrate preparation**

Corning coverglasses (#14) were cleaned by repeated dipping in boiling water followed by 95 % ethanol and dry-heat sterilized. Thin films of magnesium fluoride were deposited to λ/4 and λ/2 optical thickness (λ = 546 nm) on Corning coverglasses by Evaporated Metal Films Corporation, Ithaca, N.Y. When scanned spectrophotometrically by the manufacturer, the λ/2 films showed a maximum reflectance at 600 nm and therefore were λ/2 in optical thickness at this wavelength. These substrates were cleaned and sterilized as above.

**Optical techniques**

The interference reflexion microscope was set up as described by Curtis (1964) but using routinely available Zeiss equipment. A Photomicroscope II was equipped with a type 11 C vertical illuminator, a reflector insert carrying the aperture stop, and 40/0.85 epiachromat POL oil-immersion or 100/1.25 epiplanachromat POL oil-immersion objectives. This system provided Köhler illumination and therefore the ability to control the illuminating numerical aperture over the full range of each objective. The latter feature was essential for interpreting the complex interference image and for selecting the component of the image attributable to the thin film of medium between cell and substrate. The lever for adjusting the aperture stop in the reflector insert is calibrated to indicate the aperture diameter in mm. For the most frequently used aperture settings, the maximum angle of incidence on the specimen was determined by oiling the front surface of the objective to a glass optical flat, 1.5 cm thick. The radius
C. S. Izzard and L. R. Lochner

of the divergent cone of light striking a sheet of paper held against the lower surface of the flat was used to calculate trigonometrically the maximum angle of incidence. From this value the illuminating numerical aperture (I.N.A.) was obtained. Snell's Law furnished the maximum angle of refraction in the film of medium and in the cytoplasm for each aperture setting. These values are listed in Table 1.

Table 1. Maximum angles of incidence and refraction for different aperture settings

<table>
<thead>
<tr>
<th>Objective</th>
<th>Aperture diameter, mm</th>
<th>Angle of incidence in glass</th>
<th>I.N.A.</th>
<th>Angle of refraction in medium</th>
<th>Angle of refraction in cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/1.25</td>
<td>0.5</td>
<td>18.43°</td>
<td>0.48</td>
<td>21.00°</td>
<td>20.68°</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>33.69°</td>
<td>0.84</td>
<td>38.98°</td>
<td>38.30°</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>45.00°</td>
<td>1.07</td>
<td>53.31°</td>
<td>52.19°</td>
</tr>
<tr>
<td>40/0.85</td>
<td>0.5</td>
<td>9.46°</td>
<td>0.25</td>
<td>16.74°</td>
<td>10.58°</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>14.93°</td>
<td>0.39</td>
<td>17.00°</td>
<td>16.73°</td>
</tr>
</tbody>
</table>

The thickness of the thin films was calculated from the expression \( \Delta = \frac{2n_1d \cos \theta'}{n} \), where \( n \) is the refractive index of the thin film, \( d \) is the thickness and \( \theta' \) is the angle of refraction in the thin film. The optical path difference (\( \Delta \)) generated by the thin film was determined from the interference colours produced in white light in the interference reflexion microscope. Retardations were assigned to specific interference colours from the lists after Mönch or Quinke in the Zeiss reprint S 40-544-e (Interference colour chart according to Michel-Levy by J. Gahm).

Comparative studies on the same cell were made in transmitted light with Zeiss differential interference optics (40/0.85 achromat oil-immersion and 100/1.25 planachromat oil-immersion) and with Zeiss interference optics (Jamin-Lebedeff system 40/0.65). Optical path differences between the cell and surrounding medium (\( \Delta' \)) were measured with the latter system using de Sénarmont compensation and the extinction method. The thickness of the cell (\( d \)) was calculated from the expression \( \Delta' = d(n_1 - n_2) \), where \( n_1 \) and \( n_2 \) are the refractive indices of the cytoplasm and the medium respectively.

For the above optical techniques, monochromatic light was supplied by a 200-W/2 mercury arc with a DC power supply (Opti-Quip, Highland Mills, N.Y.) and a 546-nm Pil interference filter (Zeiss). White light (3200 °K) was provided by a 12-V/60-W tungsten source operated at 12 V and used with an 82 A filter. Heat-reflecting filters (Califlex) were used with both sources. Monochromatic images were recorded on Kodak Panatomic-X 35 mm film processed in Diafine developer (Acufine Inc., Chicago, Ill.) and white light images on Kodak High Speed Ektachrome colour transparency film with ESP-i processing to increase the ASA. A correction filter (CC 10 G) was used where necessary for the long exposures in white light with the interference reflexion microscope.

The refractive indices of the culture medium and immersion media were measured to 4 decimal places with an American Optical refractometer (\# 10402). The average refractive index of the cell cytoplasm was determined by immersion refractometry (Barer & Joseph, 1955) using Bovine Plasma Albumin Fraction V (Armour Inc., Kankakee, Ill.). A Reichert anoptral phase-contrast system was used to determine the closest match of the immersion medium to the cytoplasm. A value of 1.356 was obtained from repeated attempts.

RESULTS

Evaluation of the interference reflexion image

The principle underlying the generation of the interference reflexion image from reflexions arising at the 2 boundaries of the thin film of medium between a cell and a glass substrate has been well documented by Curtis (1964). However, the interference reflexion image produced by our optical system was more complex than would
be expected from the single thin film of medium and changed extensively as the illuminating numerical aperture (I.N.A.) of the system was varied. Fig. 3 (p. 151) illustrates these features and compares the interference reflexion images with that obtained by transmitted-light differential interference optics. Approximately 2 min were required to record the 3 images. The general outline of the cell (Fig. 3A) corresponded closely with the outline of the interference reflexion images (B, C). At low I.N.A. (0.48) a complex interference pattern was generated in this (Fig. 3B) and other cells. In

![Fig. 1. Ray diagram to illustrate the successive splitting of the incident wavefront to generate 3 reflected wavefronts coherent for interference.](image)

monochromatic light (546 nm) a series of alternating concentric maxima and minima followed the general outline of the cell. However, the intensity of an individual maximum or minimum varied as it passed around the cell. At high I.N.A. (1.07) the series of concentric maxima and minima were largely eliminated and the residual interference pattern consisted of a relatively uniform, low-intensity reflexion extending under much of the cell (Fig. 3C). Variations in the residual interference pattern consisted of darker streaks near the spreading margins of the cell and higher-intensity patches primarily under the central and non-spreading regions of the cell. This variation corresponded precisely with the variation in intensity of the maxima and minima seen at low I.N.A. In white light at low I.N.A. the most peripheral minimum produced a purple colour and the succeeding maxima and minima produced a series of interference colours which increased in order toward the centre of the cell. In contrast, these
coloured fringes were lost at high I.N.A. and the residual interference pattern generated blackish-blue, grey and white colours of the zero order.

We have interpreted the different interference reflexion images as follows. The zero-order interference pattern generated at high I.N.A. is considered to arise from the thin film of medium between cell and substrate whereas the interference pattern generated at low I.N.A. is considered to be a complex pattern generated by the thin film of medium and the cell acting as a second thin film. This interpretation is supported by the fact that the intensity of the concentric fringes is modulated in a pattern that coincides with the zero-order interference pattern and that the concentric fringes increase in order toward the presumably thicker central region of the cell. Based on this interpretation, a ray diagram for the system is given in Fig. 1 showing the reflexions at the boundaries of the 2 thin films, the refractive indices of the 4 media determined as described in the Methods, and the relative phase shifts due to reflexion. The Fresnel reflexion coefficients for the 3 boundaries, in the direction of propagation shown in Fig. 1, are listed in Table 2. Since the coefficients are small, only the primary reflexions at each boundary are included in Fig. 1 and need by considered here; secondary and subsequent reflexions arising within each film will contribute insignificantly to the interference image.

The above observations and our interpretation of them differ from those of Curtis (1964). He was aware of the possibility that the cell could act as a second thin film and accordingly looked in white light for coloured interference fringes resulting from reflexions from the far side of the cell. He reports that the only coloured fringes detectable with his optical system occurred over the nucleus and at the edge of the cell, in which case the fringes increased in order toward the edge of the cell. Both sets of fringes also were detectable in our system (see below). His observations appear to have been made primarily at low I.N.A. (0·3) and therefore under conditions at which we found the concentric, higher-order, fringes most pronounced. It was necessary, then, to provide additional confirmation of the origin of the concentric fringes and to explain their loss at higher I.N.A. The following approaches were taken.

The normal culture medium was replaced with isotonic bovine plasma albumin/salt solution mixtures of progressively higher refractive indices as described in the Methods for determining the refractive index of the cell. As the refractive index of the medium increased toward that of the cell, the contrast of the concentric fringes at low I.N.A. was reduced to a low level. This loss of contrast at matching refractive indices is consistent with the expected marked reduction in reflectivity of the cell-medium boundary at the far side of the cell. Contrast was restored as the refractive index of

<table>
<thead>
<tr>
<th>Boundary</th>
<th>Glass-medium</th>
<th>Medium-cytoplasm</th>
<th>Cytoplasm-medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresnel coefficient</td>
<td>( r_{12} = 0.0628 )</td>
<td>( r_{23} = -0.0074 )</td>
<td>( r_{34} = 0.0074 )</td>
</tr>
<tr>
<td>Calculated from the expression</td>
<td>( \frac{n_1 - n_3}{n_1 + n_3} )</td>
<td></td>
<td></td>
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</tbody>
</table>
the medium was increased above that of the cell. The contrast of the zero-order interference pattern seen at high I.N.A. was not affected in these short term medium exchanges; effects on this component of the interference pattern required long exposures to the immersion medium presumably because of the problem of exchange of medium in the space between cell and substrate.

The second approach was to calculate an expected thickness of the cell for a given concentric fringe in the interference reflexion image and to compare this with the thickness measured at the same point in the cell by transmitted-light interference microscopy (Jamin-Lebedeff system, see Methods). To ensure coincidence of the point in the cell at which thickness was measured by the 2 different techniques, the cell was photographed in monochromatic light using the Jamin-Lebedeff system set for extinction of a discrete region of the cytoplasm, and then photographed in monochromatic light at low I.N.A. (< 0·4) using the interference reflexion method (40/0·85 achromat). Comparison of the 2 photographs permitted an accurate identification of the concentric fringe coincident with the extinction region of the cytoplasm. Routinely not more than 2 min elapsed between paired photographs. The comparative thickness measurements agreed closely, especially those made in the thicker region of the cell (Table 3). The discrepancy, expressed in terms of orders for the interference reflexion method, did not exceed for the most part ± 0·5 order or half a fringe width, and never more than ± 1·0 order. Considering that slight changes in cell thickness could occur during the 2 min required to exchange the optical systems, the discrepancies are of little significance. The agreement of these thickness measurements, and the effects on the concentric fringes of varying the refractive index of the medium, support the interpretation that the concentric fringes are generated by reflexions from the far side of the cell and therefore that individual fringes represent contours of equal optical thickness in the cell.

In order to calculate the expected thickness of the cell from the interference reflexion image, it was necessary to determine the order (N) of a given fringe and whether the conditions for a minimum in monochromatic light resulted from an optical path difference (\(\Delta\)) equal to \(N\lambda\) or to \(N\lambda + \lambda/2\). The two different conditions for minima of the same order correspond respectively to whether or not a phase difference of \(\pi\) is introduced by reflexion at one of the boundaries limiting the thin film. The system illustrated in Fig. 1 and the sign of the reflexion coefficients in Table 2 show that, relative to the stronger reflexion \(r_{12}\) from the glass-medium boundary, \(r_{20}\) but not \(r_{34}\) undergoes a phase shift of \(\pi\) on reflexion. Therefore, one would predict that conditions for a minimum in the thin film of medium would occur when \(\Delta = N\lambda\) and in the cell when \(\Delta = N\lambda + \lambda/2\). The interference colours generated in white light are different for the 2 conditions: those involving a phase change on reflexion at one surface are characterized by the colours produced by a thin plate in air in reflected light; those involving no relative phase change due to reflexion are characterized by a thin plate in air in transmitted light. Accordingly, we used the colours given by Gahm for these conditions to identify the fringes (see Methods). The colour differences between the 2 conditions are most pronounced for the first-order minimum and the succeeding maximum. Thus, when \(\Delta = N\lambda\) there is a sharp transition from a red-purple through
violet to a distinct blue followed by a near white at the succeeding maximum in contrast to the less sharp transition from a purple-red through violet-purple and greenish-blue to a distinct green at the succeeding maximum when $\Delta = N\lambda + \lambda/2$. Examination of a large number of cells in white light demonstrated that the peripheral concentric minimum in monochromatic light corresponded to a first-order minimum given by $\Delta = N\lambda + \lambda/2$. From this reference minimum, $\Delta$ for any succeeding minimum could be obtained by counting fringes and the expected thickness of the cell calculated from the expression $\Delta = 2nd \cos \theta$ using $n = 1.356$ as determined by immersion refractometry. It should be noted at this point that, where trailing portions of a cell were clearly lifted away from the substrate (e.g. Fig. 5A), the first coloured fringe showed the sharp transition from red to violet characteristic of the condition for a minimum where $\Delta = N\lambda$. The observed interference colours therefore agreed with the conditions predicted from our interpretation of the complex interference pattern.

Finally the loss of the higher-order interference fringes with increasing I.N.A. has been explained in part by Curtis (1964). However, his observations relative to this point differ again from those made with our optical system. Curtis reports that by
opening the iris the (illuminating) cone angle could be increased and the image destroyed by interferences at many different angles of incidence. In contrast a residual, zero-order, interference pattern was retained at high I.N.A. with our system (Fig. 3 c). Nevertheless, we agree with his explanation, but only for the first and higher-orders of interference. Increasing the I.N.A. produces a continuous but increasingly broad range of angles of incidence on the specimen. The result is the production of a continuous series of overlapping interference patterns in which the positions of the minima are shifted progressively to thicker regions of the thin film according to the expression Δ = 2nd cos θ'. The effect is illustrated in Fig. 2 for a set of selected angles of incidence. Inspection of the figure will show that summation of the intensities of the different interference patterns will result in a relatively uniform intensity across the film and loss of distinct fringes. For small increases in I.N.A. the higher-order fringes will be lost and, as the range of angles of incidence is increased further, progressively lower-order fringes will lose contrast. Exactly this effect was observed as the I.N.A. was increased. However, for film thicknesses ranging from 0 to ca. 100 nm summation of the intensities will always result in a progressive increase in intensity when a phase change of π occurs at one surface, i.e. for the thin film of medium. Similarly a progressive decrease in intensity will occur when no relative phase change on reflexion is involved, i.e. for the cell acting as a thin film. Nevertheless, since the thickness of the cell does not fall to 100 nm (Abercrombie et al. 1971) the latter intensity differences do not apply. These points are of considerable importance, for they explain explicitly why the concentric first- and higher-order fringes are eliminated while the zero-order fringes are retained at high I.N.A. (1.07) in our optical system.

Increasing the field aperture of the interference reflexion microscope resulted in a slight but general loss of contrast in the interference reflexion image for a given I.N.A., but did not produce a sequential loss of higher-order fringes. The general loss of contrast is presumably due to the introduction of stray light into the interference image from areas outside the specimen. In practice the field diaphragm was maintained at as low a setting as possible and hence appears in some of the figures.

The interference reflexion microscope can be used therefore to examine the closeness of contact between a cell and its substrate if the illuminating numerical aperture of the system is maintained high. Since a phase shift occurs on reflexion from the cell-medium boundary of the thin film between cell and substrate, the closest contacts will appear darkest and less-close contacts progressively lighter in monochromatic light up to a separation distance of about 100 nm. The remainder of this paper describes the types of contacts between chick heart fibroblasts and glass substrates using this technique, and relates the contacts to internal structures of the cell.

Cell-to-substrate contacts and associated structures

Typical examples of chick heart fibroblasts spreading on glass substrates and photographed with differential interference optics and with the interference reflexion technique (I.N.A. = 1.07) are given in Figs. 4–6. Two of the cells were spreading both toward the top and lower right as oriented in the figures (Figs. 4 A, 5 A); the third toward the left and right (Fig. 6 A). The spreading regions comprised typical, thin,
broad sheets of cytoplasm, the leading lamellae (Ingram, 1969; Abercrombie et al. 1970), which contained few cell organelles. A non-spreading trailing portion of the cell extended toward the lower left in the first 2 cells (Figs. 4A, 5A) and the lower right in the third cell (Fig. 6A). The nucleus, partially obscured by refractile granules, was centred approximately between the spreading and trailing portions of each cell. Mitochondria appeared as refractile, somewhat elongate, sinusoidal structures in the differential interference image and were typically present in the perinuclear zone, sometimes extending into the leading lamellae (Figs. 4A, 5A, 6A).

Table 3. Determination of cell thickness

<table>
<thead>
<tr>
<th>Thickness, μm</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Cell no.</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Measured in perinuclear region</td>
</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>16</td>
</tr>
<tr>
<td>17</td>
</tr>
<tr>
<td>20</td>
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<td>24</td>
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<td>26</td>
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<tr>
<td>27</td>
</tr>
<tr>
<td>39</td>
</tr>
<tr>
<td>41</td>
</tr>
<tr>
<td>Measured in leading lamella</td>
</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>29</td>
</tr>
</tbody>
</table>

Discrepancy expressed relative to the Jamin-Lebedeff method in orders for the interference reflexion method. One order equals ca. 0.2 μm difference in thickness.

The interference pattern generated in reflected light (Figs. 4B, 5B, 6B) corresponded in general outline with the shape of the cells in the differential interference image (Figs. 4A, 5A, 6A). Small differences in the exact outline of cells in the 2 different optical images were largely associated with the distal edge of the leading lamellae (see especially Fig. 4A, B) and arose from active changes in the shape of these structures occurring during the time required to exchange optical systems. Non-spreading portions of the cell margin showed a close correspondence in the 2 optical images, e.g. the series of slender processes extending diagonally from the lower margin of the cell in Fig. 5A and B. However, where any portion of the cell was lifted well clear of the substrate there was considerable mismatch between the differential interference image and the high I.N.A. interference reflexion image. For example, the trailing portion of the cell in Fig. 5A was slightly out-of-focus and appeared to pass over the exposed surface of the following cell; the trailing portion was not detectable in Fig. 5B. The same was true for the cell in Fig. 6A and B. In both cases at low I.N.A., a series of coloured interference fringes was generated beneath the trailing portions of the cells.

In monochromatic light (546 nm) actively moving fibroblasts generated a uniform
low-intensity reflexion in the interference pattern under the leading lamellae. The intensity was consistently less than that of the reflexion from the surrounding, single, coverslip-medium boundary. The low-intensity reflexion, appearing as a dark grey shade in Figs. 4B, 5B and 6B, extended from or near the edge of the leading lamellae toward or in some cases under the nuclear region of moving cells. In white light, the same regions of the cell generated a distinct iron-grey interference colour. (The cell-to-substrate separation distances corresponding to these characteristic interference colours are given later in the Results.) Because all spreading cells consistently possessed areas that generated this iron-grey interference colour, these areas will be referred to as the close contact or grey fringe (see also Lochner & Izzard, in preparation).

In moving cells the close contact formed an uninterrupted band behind the spreading margins of the leading lamellae. The width of the uninterrupted band ranged from 2–3 μm (Fig. 5B) to 15–20 μm (Fig. 4B). Under the remainder of the cell, the close contact was dissected to varying degrees (Figs. 3C, 5B) or merged into a reflexion of higher intensity which approached that of the surrounding coverglass-medium boundary (Figs. 4B, 6B). Dissection of the close contact appeared as either an irregular piebald pattern (Fig. 5B) or a more linear pattern (Fig. 3C) of reflexions of higher intensity than those from the surrounding coverglass-medium boundary. The high intensity reflexions generated white to yellowish interference colours and therefore corresponded to areas of distinctly greater cell-to-substrate separation than the close contact. Dissection of the close contact was typically more extensive under the nuclear and perinuclear regions of the cell. As spreading of the leading lamellae and cell movement ceased, dissection of the close contact increased, first under the nuclear region and then progressively under the leading lamellae, until only a narrow band of close contact, often highly dissected, remained at the tips of the leading lamellae. In these cases the interference colours generated in white light ranged from a pale grey through white under the leading lamellae to a yellow under the nucleus demonstrating that stationary cells were well separated from the substrate over much of their surface (see Lochner & Izzard, in preparation).

The closest contacts between cell and substrate appeared as very low intensity streaks in monochromatic light (Figs. 3C, 4B, 5B, 6B). The same regions generated a blackish-blue interference colour in white light. The very close contacts ranged from 0.25 to 0.5 μm in width and 2 to 10 μm in length, and were located predominantly at the periphery of the cell. Under the leading lamellae the streaks were oriented with their long axes approximately parallel to the direction of spreading and were distributed almost exclusively within the undissected zone of close contact, that is within a zone extending 3 μm (Fig. 5B) to 20 μm (Fig. 4B) behind the edge of the leading lamella. Similar blackish-blue streaks were located under the distal ends of extended, non-advancing, regions of the cell periphery, e.g. under the diagonally oriented extension from the lower edge of the cell in Fig. 5B, and under the trailing portions of many cells (Fig. 4B). Individual or small clusters of blackish-blue streaks were found occasionally under the nuclear or perinuclear regions of the cell. These very close contacts will be referred to as focal contacts throughout the remainder of this paper.

The focal contacts were located under the peripheral ends of discrete cytoplasmic
fibres visible in the differential interference image (compare Figs. 3A and C, 4A and B, 5A and B, 6A and B). The same fibres were positively birefringent with respect to their long axis. The width of the fibres was 0.25-0.5 μm, the peripheral ends of some fibres splaying out to 2 μm in width. In the latter case the shape of the end of the fibre corresponded precisely with that of the associated focal contact (e.g. see at the lower-right margin of the cell in Fig. 5A, B). The length of the fibres varied; in some cells it was approximately the same as but not markedly less than that of the associated focal contact (Fig. 5A, B). However, in many cells the fibres extended from the focal contacts through the leading lamellae into the perinuclear region. The fibres either ran parallel to the substrate and lay for much of their length in the plane of optical section (Fig. 6A, B), or ran obliquely from the focal contact and could be followed by focusing deeper into the perinuclear regions, as was the case in Fig. 4A, B. Similar fibres were frequently associated with the focal contacts under non-advancing or trailing portions of the cell (see lower-right in Fig. 5A, B).

Cytoplasmic fibres that ran more or less parallel to the substrate had a distinct effect on the cell-to-substrate separation distance under the nuclear and perinuclear regions of the cell. Where the close contact was dissected by areas of distinctly greater separation distance, these areas were usually elongate and located between pairs or groups of parallel fibres (Fig. 3A, C). Where the close contact was more continuous or merged into an area of slightly greater separation distance under the centre of the cell, distinct ridges protruding toward the substrate could be detected as darker lines in the interference reflexion image. The ridges showed a precise spatial correlation with cytoplasmic fibres (Fig. 6A, B). The intensity of the reflexion corresponding to the ridges did not fall as low as that from the focal contacts. Such ridges could be followed in some instances from focal contacts at one end of the cell to similar contacts at the other end of the cell (e.g. group of ridges passing under the nucleus in Fig. 6B).

Occasionally the cell surface extending posteriorly from a focal contact generated an elongate high-intensity reflexion that contrasted strongly with the reflexion from the close contact beneath the leading lamella. In white light the reflexion generated white to yellowish interference colours. These elongate spacings were seen only beneath portions of the cytoplasmic fibre that extended posteriorly from the focal contact (compare Fig. 4A and B) and were usually associated with fibres that ran obliquely into the cytoplasm.

Reflexions from the nucleus produced readily recognizable sets of coloured interference fringes at low I.N.A. as described by Curtis (1964). Where the lower surface of the nucleus appeared to be flat from the form of the interference fringes and was close to the lower surface of the cell, the nucleus could still be detected in the interference image even at high I.N.A. (1.07) (Fig. 6A, B). Similarly, the strong reflexions from the refractile granules in the perinuclear region of the cell produced small sets of circular fringes at low I.N.A. that remained detectable at high I.N.A. (Fig. 3A–C). Again, these structures generated coloured, higher-order, interference colours in white light. Mitochondria did not contribute significantly to the interference reflexion image even at low I.N.A.
Appearance of the lamellipodium in the interference reflexion image

The edge of the leading lamella has been shown in chick heart fibroblasts to undergo a series of alternating protrusions and withdrawals that result in a net advance of the margin of the cell (Abercrombie et al. 1970). This mobile sheet of cytoplasm, the lamellipodium, had previously been seen in side-view to extend usually parallel to the substrate but also not uncommonly free into the medium at an acute angle to the substrate. Upward deflexion of the lamellipodium from either of these positions generates the marginal ruffling activity (Ingram, 1969; Harris, 1969) that accompanies cell spreading. Lamellipodia can be detected readily in the interference reflexion image and their motile behaviour followed directly without recourse to time-lapse cinematography. Because of the relatively rapid movement of the lamellipodia, the comparative photographs in Fig. 7 were taken with an alternative optical system in which only 10 s were required between the initiation of the 2 exposures (see Lochner & Izzard, in preparation). As illustrated in Fig. 7B, the close contact does not extend to the extreme edge of the cell across the whole margin of the leading lamella. Instead, the lower-intensity reflexion of the close contact passes abruptly into a reflexion of higher intensity than that of the background reflexion from the glass-medium boundary. The high-intensity reflexion extends up to 3 \( \mu \text{m} \) ahead of the close contact, but lies within the outline of the cell in the differential interference image (Fig. 7A). This high-intensity reflexion was generated by the lamellipodium extending parallel to the substrate but more widely separated from it than the following close contact area. Such lamellipodia generated white to yellow interference colours close to the zero-order maximum. In some cells the base of the lamellipodium, and therefore the distal edge of the close contact, coincided precisely with the distal edge of a distinctly shadow-cast structure running across the leading lamella in the differential interference image (Fig. 7, and see Lochner & Izzard, in preparation). This structure represents a local thickening of the leading lamella (Lochner & Izzard, unpublished results). The width of lamellipodia varied significantly at different points across the leading lamellae (Figs. 4B, 5B, 6B). In actively spreading cells lamellipodia were commonly 3–5 \( \mu \text{m} \) in width but occasionally extended up to 14 \( \mu \text{m} \) in width. In these wider lamellipodia, the high-intensity reflexion at their base frequently passed into a series of minima and maxima extending toward their tip. In white light the minima showed first- and higher-order interference colours demonstrating that the lamellipodia were inclined at an angle to the substrate. The colours of the first minimum were characteristic of the condition where \( \Delta = N\lambda \), i.e. of the thin film of medium. The protrusion and withdrawal of the lamellipodium and its deflexion away from the substrate are described elsewhere (Lochner & Izzard, in preparation).

Measurement of cell-to-substrate separation distance

In a preliminary report of this work (Lochner & Izzard, 1973) and quoted by Abercrombie & Dunn (1975) the cell-to-substrate separation distances were given as follows: focal, very close contacts 45 nm or less; uniform close contact or grey fringe ca. 80 nm. These values were obtained by comparing the observed hues of the zero-
order colours with those given for thin plates in reflected incandescent light involving a phase change of \( \pi \) at one surface (see Methods). The observed colours of blackish-blue for the very close contacts and iron-grey for the uniform close contact were assigned retardations of 120 and 210 nm, respectively. Although it was necessary to increase the I.N.A. towards 0.84 in order to minimize the concentric fringes, the cell-to-substrate separation was calculated from these retardation values assuming normal incidence. Both separation distances are significantly larger than those determined for chick heart fibroblasts from densitometry of interference reflexion micrographs by Curtis (1964) and from electron micrographs by Abercrombie et al. (1971) (see Table 4). For reasons given in the Discussion, we believe that the densitometric method used by Curtis (1964), although sound in theory, is subject to practical errors resulting from the reflexions at the far side of the cell. Our initial attempt to measure retardation was equally subject to error for the following reasons. Firstly it was extremely difficult to determine the exact hue of grey in the zero order. Secondly, because the amplitude of the wavefront reflected from the glass-medium boundary is 8.5 times greater than that from the medium-cell boundary (see Table 2), complete destructive interference will not occur at any wavelength. The result is a set of interference colours saturated with white light, a situation directly analogous to the weakly coloured fringes generated from a thin film in transmitted light. The second problem is especially critical when dealing with the zero-order colours, for example a blackish-blue interference colour for matched amplitudes could appear iron-grey in the system used here.

To overcome both problems bias retardations of known values were added to the retardations generated by the thin film of medium between cell and substrate so that the interference colours would be shifted to the distinctive colours of the first-order minimum. This was accomplished by first coating the coverslips with a thin film of magnesium fluoride (see Methods) and culturing the cells on this film, the assumption being that the cells would attain the same separation distance from magnesium fluoride as from glass. Magnesium fluoride was selected for its insolubility and because it would not introduce phase changes upon reflexion since its refractive index (1.38) is intermediate between those of glass and the culture medium. A bias retardation of \( \lambda/2 \) was required for the focal and close contacts and of \( \lambda/4 \) for the areas of greater separation under the centre of the cell. These retardations were achieved by using films of \( \lambda/2 \) and \( \lambda/4 \) optical thickness at 546 nm (in practice the thicker films were \( \lambda/2 \) at 600 nm – see Methods). The efficacy of the method is demonstrated in Figs. 8 and 9. With a bias retardation of \( \lambda/2 \) at 546 nm (Fig. 8) the contrast relationships of the different contact regions were reversed relative to those without a bias retardation (e.g. Fig. 7B). Thus the elongate, focal areas of very close contact generated high-intensity reflexions. They were surrounded by a uniform area of lower intensity but higher than that of the background reflexion. The piebald areas under the cell produced reflexions of lower intensity than the background, sometimes passing through a minimum toward the succeeding maximum. Addition of \( \lambda \) bias retardation (Fig. 9) restored the contrast relationships in monochromatic light to those seen without bias retardation.

The retardation values that we assigned to the observed interference colours without
bias retardation and the separation distances calculated from those values are listed for the focal contacts, close contacts, and the areas of greater separation from the substrate in Table 4. These values are compared in the same columns with the separation distances for similar regions (see Discussion) given by Curtis (1964) and Abercrombie et al. (1971) and with the retardation values calculated by us for these distances.

Table 4. Predicted retarding and interference colours with bias retardation

<table>
<thead>
<tr>
<th></th>
<th>(d), nm</th>
<th>(\Delta + 0\text{-nm bias})</th>
<th>(\Delta + 273\text{-nm bias})</th>
<th>(\Delta + 600\text{-nm bias})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Focal contacts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Our value</td>
<td>45</td>
<td>120 Blackish-blue</td>
<td>393 Straw yellow</td>
<td>720 Green</td>
</tr>
<tr>
<td>Curtis (1964)</td>
<td>10</td>
<td>27 Black</td>
<td>300 White</td>
<td>627 Blue</td>
</tr>
<tr>
<td><strong>Close contacts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Our value</td>
<td>79</td>
<td>210 Iron-grey</td>
<td>483 Red-brown</td>
<td>810 Yellow</td>
</tr>
<tr>
<td><strong>Areas of greater separation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Our value</td>
<td>112–138</td>
<td>300–369 White to yellow</td>
<td>573–642 Violet to blue</td>
<td>900–969 Yellow-orange to orange-brown</td>
</tr>
<tr>
<td>Curtis (1964)</td>
<td>30–50</td>
<td>80–134 Black to blackish-blue</td>
<td>353–407 Yellow to straw yellow</td>
<td>680–734 Blue-green to greenish-white</td>
</tr>
<tr>
<td>Abercrombie et al. (1971)</td>
<td>100</td>
<td>267 Iron-grey to colourless</td>
<td>540 Red-purple to violet</td>
<td>867 Yellow to yellow-orange</td>
</tr>
</tbody>
</table>

See text for explanation of Table.

\(d\) = separation distance between cell and substrate.

\(\Delta\) = optical path difference generated by \(d\).

Normal incidence has been assumed throughout the Table.

* Lower value of \(d\) measured by us from published micrographs.

** Both values of \(d\) measured by us from published micrographs.

The total retardations with biases of 273 and 600 nm are listed respectively in the following columns together with the expected interference colour in each case. Normal incidence has been assumed throughout Table 4. In practice it was necessary to vary the I.N.A. up to 0.84 in order to evaluate the effect on the colours of the concentric fringes generated by cell thickness. The 100/1.25 planachromat objective was used for this work.
The focal contacts appeared white with a bias retardation of 273 nm and generated a blue colour with the more sensitive bias retardation of 600 nm. In both cases the colours are distinctly different from those expected on the basis of our previous upper limit of 45 nm separation but correspond to the expected colours based on the separation distances of 10-15 nm given by Curtis (1964) and Abercrombie et al. (1971).

The close contact, where present as an uninterrupted zone behind the spreading margin of the leading lamella (Fig. 4B) and as a dissected contact beneath the centre of the cell (Fig. 5B), generated a weak yellow hue or a blue-green colour respectively with bias retardations of 273 and 600 nm. The observed colours are closest to those predicted from the lower separation distance taken from Abercrombie et al. (1971) and distinct from those based on our earlier value without bias retardation. A value of ca. 30 nm is appropriate for the close contact.

Areas of the cell that were separated widely from the substrate differed in extent, as described above, depending on whether or not the cell had been actively spreading. Nevertheless, the same range of interference colours was generated on the MgF₂ films irrespective of the extent of the areas of wide separation. Thus, with a bias retardation of 273 nm red-purple, violet or blue colours corresponded to the peaks of cell-to-substrate separation and, with bias retardations of 600 nm, the peaks produced yellow-orange or orange-brown colours. In both cases, the colours corresponded well with those expected from the upper limit given by Abercrombie et al. (1971) and from the distances estimated by us without bias retardation. The colours differed significantly from those based on Curtis's (1964) values. However, it is not clear to what extent Curtis could recognize these areas in his images (see Discussion). Therefore the wider separation distances ranged from 100 to 140 nm.

Lamellipodia extending parallel to the substrate produced a low-intensity reflexion in monochromatic light with 273 nm bias retardation (Fig. 8) in contrast to the high-intensity reflexion without bias (Fig. 7B). In white light a purple interference colour (1st order) was generated with this bias corresponding to a separation distance of about 100 nm.

Use of the 600-nm bias films eliminated an alternative explanation for the different intensities generated by the focal and close contacts in monochromatic light. If the refractive index of the cell surface were higher in the region of the focal contact than in the surrounding areas, this would produce a stronger reflexion and bring about more complete destructive interference. The possibility is strengthened by the association of the dense cytoplasmic fibres with the focal contacts. However, since different interference colours were produced by the 2 contacts with 600-nm bias retardation, different optical path differences were involved. Local variations in the refractive index of the thin film between cell and substrate cannot account for the retardation differences (see Discussion) and therefore the differences in separation distance for the focal and close contacts are real.
DISCUSSION

Interference reflexion microscopy (Curtis, 1964) has been re-evaluated as a technique for examining the closeness of contact between cultured cells and planar glass substrates. Our results show that the interference reflexion image is more complex than can be accounted for solely in terms of the thin film of medium between cell and substrate. At low I.N.A. a pronounced set of concentric, first and higher order, interference fringes are produced. Abercrombie & Dunn (1975) have drawn attention to these fringes. We have presented here the experimental basis for our personal communication to them, that these concentric fringes originate from reflexions from the cell-medium boundary at the far side of the cell. The fringes represent, therefore, contours of equal optical thickness in the cell. Certain features in Curtis's monochromatic images (1964), that show a similarity to these higher-order concentric fringes, are discussed below. By examining the effect of varying the I.N.A. of the reflecting system, we found that the concentric fringes due to cell thickness could be eliminated at high I.N.A. (> 1.0) leaving a residual, zero-order, interference pattern attributable to the thin film of medium between cell and substrate. The theoretical reason for the loss of contrast in first- and higher-order fringes at increasing I.N.A. has been discussed above and was consistent with observation. From this theoretical treatment it was clear that differences in the thickness of the thin film of medium over the range 0 to ca. 100 nm would always appear as interpretable relative intensity differences in the interference reflexion image. The importance of this point cannot be overemphasized because, under the operational conditions defined in the Results, the interference reflexion microscope now can be used effectively to examine the relative closeness of contact between different regions of the cell surface and a planar substrate.

Interference reflexion microscopy has demonstrated that 2 distinct types of cell-to-substrate contact are formed when chick heart fibroblasts spread on glass substrates. Varying proportions of the undersurface of cells are occupied by broad regions of uniformly close contact representing a separation distance of ca. 30 nm. The uniform spacing of these close contacts extends from the base of the lamellipodium under the leading lamella, but may be broken by areas of greater separation (100-140 nm). Focal, very close contacts, separated by 10-15 nm from the substrate, are distributed within the area of close contact under spreading portions of the cell and under the distal ends of non-spreading and trailing portions of the cell. The origin of this distribution is described in a following paper (Lochner & Izzard, in preparation).

The system of localized focal contacts and associated cytoplasmic fibres, demonstrated here in living chick heart fibroblasts, appears to be directly equivalent to the system of close contacts (30 nm or less), associated plaques of amorphous electron-dense material and contiguous tracts of oriented microfilaments described from electron-microscope studies of the same cells by Abercrombie et al. (1971). Although in the present study plaques could not be distinguished at the light-microscope level from contiguous microfilament tracts, it is not unreasonable to conclude that those portions of the cytoplasmic fibres overlying the focal contacts are equivalent to the
plaques recognizable at the ultrastructural level. Similarly, the portions of the cytoplasmic fibres extending from these plaques toward the perinuclear region of the cell undoubtedly are equivalent to the tracts of oriented microfilaments described by Abercrombie *et al.* (1971). This latter conclusion is supported by the fact that the same cytoplasmic fibres are positively birefringent with respect to their long axes, as would be expected for an elongate structure consisting of parallel microfilaments. The common identity of the system of focal contacts and fibres detectable in living cells with that seen in electron-microscope preparations is further emphasized by the following detail. Abercrombie *et al.* (1971) pointed out that the posterior end of a plaque tends to be lifted away from the substrate. The same detail was seen in the interference reflexion image as an elongate high-intensity reflexion extending beneath the cytoplasmic fibre from the posterior end of some of the focal contacts. In addition, Abercrombie *et al.* (1971) state that in chick heart fibroblasts regions of the cell surface separated by 30 nm or less from the substrate occupy approximately 25% of the undersurface of the leading lamella and calculate that there should be between 10 and 100 focal contacts beneath each cell. We can agree in general with these estimates, but have not made detailed counts because the number of focal contacts per cell varies not only with the width and number of the leading lamellae per cell but also with the previous motile history of the cell (see Lochner & Izzard, in preparation). Abercrombie *et al.* (1971) did not describe as such the uniform close contacts under the leading lamellae of chick heart fibroblasts and our values for these areas in Table 4 were taken from their micrographs.

Focal regions of very close cell-to-substrate contact have been described among a limited number of other fibroblast-like cells from electron-microscope sections (Cornell, 1969; Brunk *et al.* 1971). In the latter case the focal contacts were associated with dense accumulations of microfilaments in the adjacent cytoplasm. Similarly, focal regions of implied close contact and of adhesion have been described beneath cultured cells by examining respectively replicas of the undersurface of intact cells (Revel & Wolken, 1973) and of the substrate after mechanical removal of the cells (Revel, Hoch & Ho, 1974). In the former case, the implied close contacts were distributed along and especially at the ends of bundles of microfilaments in BHK cells.

Ambrose (1961) used a different optical approach with a stated vertical resolution of 50 nm to examine the closeness of contact between cultured cells and glass substrates, namely the surface-contact microscope. In the more sensitive dark-field mode, he described small regions of closest contact distributed both under the leading lamellae and under the remainder of the cell in chick heart fibroblasts. However, as illustrated, these small areas of contact were oriented with their long axes perpendicular to the direction of spreading and movement in contrast to the parallel orientation of the focal contacts described here. He states that the contacts were in continual flux and interprets this feature in terms of propagated small-scale undulations, the contacts, across the lower surface of the cell. Whatever the nature of the features observed by Ambrose (1961), they are clearly distinct from the focal contacts detected with the interference reflexion microscope, not only in distribution and orientation, but in their
Cell-to-substrate contacts in fibroblasts

fluxes since the focal contacts once formed remain stationary on average for 4.2 min in rapidly advancing cells (Lochner & Izzard, 1973, and in preparation).

Among other fibroblast-like cells evidence for the presence of broad close contacts is indirect and limited. However, Revel & Wolken (1973) described broad areas of low relief in their replicas of the undersurface of L cells and implied from the inability of Thorotrast to penetrate beneath such areas that they were in close contact with the substrate. Similarly, Revel et al. (1974) found that antibovine globulin did not penetrate readily beneath BHK cells. Exclusion of the tracers from under the cells may well have resulted from their inability to penetrate rapidly into a ca. 30-nm space or close contact. We should point out here that focal contacts and close contacts, generating blackish-blue and iron-grey interference colours respectively, have been observed beneath spread L cells using the interference reflexion technique (Izzard & Lochner, unpublished data). In contrast Brunk et al. (1971) found that, except for focal very close contacts, human glial cells in stationary monolayer culture were separated from the substrate by a system of wide ‘arches or vaults’ into which Thorotrast (15–30 nm particle diameter) readily penetrated. However, monolayered chick heart fibroblasts, while conserving the focal contacts, lose the close contacts and become separated by distances of ca. 100 nm from the substrate (Lochner & Izzard, in preparation).

The micromanipulation studies of Harris (1973) and earlier workers located the principal adhesions between a wide variety of cultured cells and their substrates to relatively narrow areas near the cell margins and especially under the leading lamellae. The distribution of focal contacts described here conforms to these adhesive areas. Abercrombie & Dunn (1975) have discussed sound lines of evidence for the adhesive properties of the focal contacts of chick heart fibroblasts, namely that the focal contacts remain stationary relative to the substrate as the cell moves (Lochner & Izzard, 1973, and in preparation), that the attachment points of retraction fibres coincide with the focal contacts in the spread cell, and perhaps most significantly that focal contacts are not separated by one cell passing between another cell and its substrate (see also Armstrong & Lackie, 1975). To these lines of evidence can be added the observation that only focal contacts persist in monolayers and in stationary, isolated, chick heart fibroblasts (Lochner & Izzard, in preparation). Therefore, these contacts alone are sufficient to transmit tension in the cell to the substrate and maintain the cell in a spread condition. In addition, the effects on cell-to-substrate separation distances of cytoplasmic fibres extending beyond the focal contacts are consistent with the fibres being under tension. Thus the lower surface of the cell was raised into ridges beneath fibres running parallel to the substrate (Fig. 6A, B) or was lifted distinctly further away from the substrate immediately behind focal contacts associated with obliquely oriented fibres. (Interpretation of the latter feature assumes that the cell surface was adherent over a longer distance to the fibre than it was to the substrate.) Collectively the evidence is good for the cytoplasmic fibres being under tension and the focal contacts being sites of adhesion. The potential significance of the close contacts in adhesion and movement will be discussed later (Lochner & Izzard, in preparation).

In general terms our account of the relative closeness of contact between cell and substrate agrees with that of Curtis (1964), namely that contact is closer and more
uniform under the peripheral regions of the leading lamellae and separation is greatest under the nuclear region of the cell. However, the morphology and distribution of the closest contacts described by Curtis and by us differ. We believe the differences arose respectively from the use of low as opposed to high I.N.A. For example, Curtis (1964) states that frequently the closest contacts (regions of 10-nm separation) form narrow bands either very close to the front edge of the cell or a little farther back but parallel to the leading edge of the cell. The I.N.A. of his system was 0.3 and, at I.N.A. ranging from 0.84 and down, similar dark interference bands occurred in our system (Figs. 3B, 7B). However, whereas Curtis (1964) describes the dark bands as black in white light, we found them to be coloured. The most peripheral band showed the colour sequence corresponding to an optical path difference (Δ) of 1.5 Å and therefore that to be expected from the cell itself acting as a thin film. In these terms, Δ = 819 nm for the peripheral minimum and, if one allows for the optical path difference introduced by a thin film of medium corresponding to the close contact (ca. 80 nm, Table 4 and Results), the calculated thickness of the cell at this point is 272 nm, a value well within the range reported from electron-microscopical studies (Abercrombie et al. 1971). Similarly, Curtis (1964) considers that most cells have a narrow band of 10–20 nm separation running around the edge of the cell. The band appears to be equivalent to the dark fringe at the left edge of the cell in Fig. 7B. Again, in our optical system such fringes were coloured in white light and showed the sharp transition from red-purple to violet characteristic of the first-order minimum for the thin film of medium. (More peripheral fringes seen at lower I.N.A. exhibited higher-order interference colours indicating that the edge of the cell was lifted away from the substrate as noted by Curtis.) The discrepancy in the observations in white light can be explained as follows. The bands, both at the front and along the side of the cell, are frequently less than 1 μm in width and, even with the higher-power objective used in the present work, it was difficult to observe any colour separation in bands of this width. However, where such narrow bands expanded in width as they passed around the edge of the cell, the colour separation became pronounced especially with the 100/1.25 objective. Therefore, we conclude that the closest contacts consist solely of the focal contacts seen clearly at high I.N.A. and do not form narrow bands parallel to the leading edge or sides of the cell.

Over the main part of the cell body Curtis (1964) considers that the surface is thrown into either a series of parallel furrows and ridges, 2–5 μm wide and oriented parallel to the direction of movement, or into a more confused pattern of furrows in more rounded cells. Although we observed parallel ridges under the cell at high I.N.A. (Fig. 6B), the ridges were only 0.25–0.5 μm wide, coincided with cytoplasmic fibres, and did not tend to follow the outline of the cell as did the ridges illustrated by Curtis (1964). The location and spacing of the ridges and furrows in his low I.N.A. monochromatic micrographs suggest strongly that they represented higher-order fringes attributable to the optical thickness of the cell.

The cell-to-substrate separation distances calculated from the interference colours produced with bias retardations agreed well with those obtained from the electron-microscope studies of Abercrombie et al. (1971), despite the use of different substrates.
Cell-to-substrate contacts in fibroblasts

For reasons given in the Results our earlier values are considered to be in error (Lochner & Izzard, 1973). The accuracy of our current measurements is limited by the retardation values for a given interference colour, e.g. the first-order blue expected for the focal contacts with 600-nm bias covers the retardation range 627-640 nm and therefore does not distinguish between separation distances of 10-15 nm (Table 4). In this context the method is good for distinguishing differences of the order of 10 nm or more. Although there is evidence for the presence of microprecipitates between solid substrates and the apposed surface of cultured cells (Brunk et al. 1971; Revel & Wolken, 1973), such precipitates could increase the refractive index of the thin film above that of the medium by only < 0.02 units and still present conditions in which a minimum is given by $\Delta = N\lambda$ (unless the refractive index of the precipitates was higher than that of glass). Therefore the presence of these materials would produce < 2% error in our calculations of the separation distances. Curtis's method (1964) for obtaining cell-to-substrate separation distances from reflectivity measurements is capable of considerable accuracy, as he evaluates. However, since the method requires that the specimen be illuminated by collimated light at near normal incidence, low I.N.A. must be used. As we have demonstrated here, the interference reflexion pattern is complex under these conditions and, apart from the difficulties in identifying the cell-to-substrate contacts at low I.N.A., any reflectivity measurements will include the additional information generated by the cell acting as a second thin film. One can estimate simply by the use of vector diagrams that calculations of the separation distance for the close contact from reflectivities measured where a minimum for cell thickness coincides with the close contact will be 25% too low. In this context the interference reflexion microscope is not applicable at the present to absolute measurements of cell-to-substrate separation distances but, as demonstrated here, is invaluable at high I.N.A. for determining relative separation distances under a cell and detecting changes in these distances with time. Accordingly we have followed the formation of new cell-to-substrate contacts during cell movement and these data are to be presented elsewhere (Lochner & Izzard, in preparation).

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REFERENCES


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Cell-to-substrate contacts in fibroblasts
Fig. 4. Chick heart fibroblast (A) in differential interference optics (100/1.25) and (B) in interference reflexion optics (100/1.25) at I.N.A. = 1.07. Time elapsed between (A) and (B) 5 min. Leading lamellae were spreading toward the top and lower right; trailing portion of the cell extends to lower left. The reflexion under the leading lamellae in (B) is uniform and lower in intensity than the background and corresponds to the close contact. The focal contacts (dark streaks) are located under the leading lamellae within the area of close contact and under the distal end of the trailing portion of the cell (B). Note that there is a precise spatial coincidence of the focal contacts under the leading lamellae (B) with cytoplasmic fibres of the same length or with the distal ends of longer fibres (A). The long focal contact in the centre of the upper leading lamella merges with an elongate higher intensity reflexion produced by a distinctly wider separation distance (B). The nucleus was centred among the refractile granules in (A) but did not contribute to the high I.N.A. image (B). See text for further details. Scale bar 10 μm.
Cell-to-substrate contacts in fibroblasts
Fig. 5. Chick heart fibroblast (A) in differential interference optics (100/1·25) and (B) in interference reflection optics (100/1·25) at I.N.A. = 1·07. Time elapsed between (A) and (B) 2 min. The close contact (B) extends as a dissected but uniform intensity reflection under the broad leading lamellae and centre of the cell. The high intensity reflexions correspond to areas of wider separation (100-140 nm). The focal contacts (B) are few in number and located near the margin of the leading lamella. Each focal contact in (B) coincides with a cytoplasmic fibre in (A). Note the distinctly more refractile and sinusoidal form of the mitochondria in the perinuclear region. The trailing portion of the cell was out of focus in (A) and did not contribute to the image in (B). Scale bar 10 μm.
Cell-to-substrate contacts in fibroblasts
Fig. 6. Chick heart fibroblast (A) in differential interference optics (100/1·25) and (B) in interference reflexion optics (100/1·25) at I.N.A. = 1·07. Time elapsed between (A) and (B) 3 min. The cell was spreading primarily to the left. Long cytoplasmic fibres lying parallel to the substrate were prominent in this cell (A). Focal contacts in (a) coincide with the peripheral ends of these fibres. Ridges on the undersurface of the cell appear as darker lines extending from the focal contacts in (B) and coincide with the fibres in (A). The nucleus lying close to the lower edge of the cell in (A) contributes to the high I.N.A. image in (B). Scale bar 10 μm.
Fig. 7. Chick heart fibroblast (A) in differential interference optics (100/1·25) and (B) in interference reflexion optics (100/1·25) at I.N.A. = 0·84. Time elapsed between (A) and (B) 10 s. The leading lamella was spreading toward the top. The lamellipodium generates a high intensity reflexion ca. 3 μm wide at the margin of the leading lamella in (B). The free edge and base of the lamellipodium in (B) coincide respectively with the outline of the cell and with the front of a shadow cast thickening in (A). Note the coincidence of the focal contacts in (B) (dark streaks parallel to direction of spreading) with fibres in (A). Two minima in (B) run perpendicular to the direction of spreading close to the edge of the leading lamella. See text for explanation. Scale bar 10 μm.

Fig. 8. Chick heart fibroblast cultured on magnesium fluoride film, λ/4 optical thickness at 546 nm, bias retardation 273 nm. Interference reflexion optics (100/1·25) at I.N.A. = 0·84. Note reversed contrast of focal contacts, surrounding close contact, and piebald patches of wide separation relative to image without bias retardation (Fig. 7B). At top left edge of the cell a lamellipodium appears in reversed contrast. See text for explanation. Scale as for Fig. 7.

Fig. 9. Chick heart fibroblast cultured on magnesium fluoride film, λ/2 optical thickness at 600 nm, bias retardation 600 nm. Interference reflexion optics (100/1·25) at I.N.A. = 0·84. The leading lamella extends toward the top. Focal contacts appear as black streaks with this bias retardation. The close contact was not pronounced in this cell but appears as grey tones around the focal contacts. See text for explanation. Scale as for Fig. 7.
Cell-to-substrate contacts in fibroblasts