



## **Silicone Rubber Substrata: A New Wrinkle in the Study of Cell Locomotion**

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## Silicone Rubber Substrata: A New Wrinkle in the Study of Cell Locomotion

**Abstract.** *When tissue cells are cultured on very thin sheets of cross-linked silicone fluid, the traction forces the cells exert are made visible as elastic distortion and wrinkling of this substratum. Around explants this pattern of wrinkling closely resembles the "center effects" long observed in plasma clots and traditionally attributed to dehydration shrinkage.*

The exertion of a rearward traction force upon an external substratum is central to any kind of crawling locomotion, such as that undergone by tissue cells and amoebae. Many workers have studied the cytoplasmic actomyosin networks believed to generate these forces (1), but we still know very little about how, where, and with what strength these traction forces are exerted so as to propel individual cells. The main difficulty is that these forces are too weak and are exerted over too small an area to be studied by conventional techniques, even including micromanipulation. Although James and Taylor (2) were able to use the bending of calibrated micro-needles to measure the contractility of whole sheets of fibroblasts stretched between bone fragments, such methods are not applicable to the exertion of traction itself, much less that of individual cells.

An alternative approach is to culture cells directly on the surface of some elastic material weak enough to be visibly distorted by the small traction forces by which cells crawl. Then, if the elastic properties of the substratum are known or can be measured, the forces exerted by individual cells can be read out continuously and nondestructively from the distortions they produce in this substratum. One of us introduced this approach several years ago in a study of the distortions and birefringence produced in thin plasma clots by cultured fibroblasts (3). Recently, another worker measured very small shearing forces by the birefringence produced in gelatin (4).

But both these protein substrata have serious drawbacks. Clottable plasma is highly unstable and the elasticity of clots tends to be unpredictable, while gelatin is apt to liquefy. Still more serious is the susceptibility of protein sheets to biochemical alteration by the cells, the results of which could be misinterpreted as changes in cell contractility. In particular, Paul Weiss postulated long ago that growing cells dehydrate protein networks around them, causing local shrinkage and, in turn, stretching the surrounding network (5). This explanation for the radial orientation of clots around explants (Weiss's classic

two-center effect) has become accepted as if it were a proven fact.

So an elastic substratum for studying traction should be as inert as possible to biochemical change, especially shrinkage. It should also be nontoxic, transparent, and ideally should permit observations of the cells' contacts by interference reflection microscopy, while showing sufficiently little strain-induced birefringence not to interfere with polarized light observations of the cells themselves. We announce here the development of silicone rubber culture substrata having all of these properties.

Silicone rubber can be made by cross-linking linear polymeric chains of polydimethyl siloxane (silicone fluid) under the influence of certain free radical catalysts, strong acids, or heat (6). Commercially manufactured silicone rubber sheets (7), intended for use in heart-lung machines, proved to be too thick and rigid to be detectably distorted by most fibroblasts. So to acquire sufficiently thin and elastic silicone rubber sheets it was necessary to synthesize them ourselves.

After testing a variety of chemical and physical methods for forming the exceptionally thin layers of rubber needed, we found a surprisingly simple method that yields reproducible results. Brief ex-

posure of silicone fluid to a flame cross-links only its outermost layers. This has the double advantage that the rubber "skin" can be made very thin (about 1  $\mu\text{m}$ ), while the un-cross-linked fluid beneath it serves as a lubricant.

The silicone rubber substrata used in the work reported here were made by the following procedure: 60,000 centipoise silicone fluid (8) is first spread in a relatively thick layer onto one surface of a cover slip. This surface is then turned face down into the top of a Bunsen-burner flame for 2 seconds. Timing is critical and this step may take practice. During flaming, a sheen of tiny wrinkles forms on the fluid surface, and if the cover slip is withdrawn as soon as these are seen, they will reflatten to form the desired smooth elastic surface. The burner should be adjusted so that the flame is low and oxygenated barely enough to prevent it being yellow and smoky at the top. By shortening the exposure to the flame and by starting with less viscous silicone fluids, one can make rubber layers that are even weaker. If very thick layers are needed, the entire fluid layer can be cross-linked by heating to 350°C for 20 minutes on a hot plate.

After preparation, these cover slips were placed in petri dishes containing Dulbecco-Vogt's modified Eagle's medium, buffered with Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] and supplemented with 10 percent fetal calf serum. Tissue cells were then plated out onto these surfaces either as explanted fragments from embryonic chicks or as trypsinized suspensions. So far, we have successfully cultured on these substrata embryonic heart fibro-

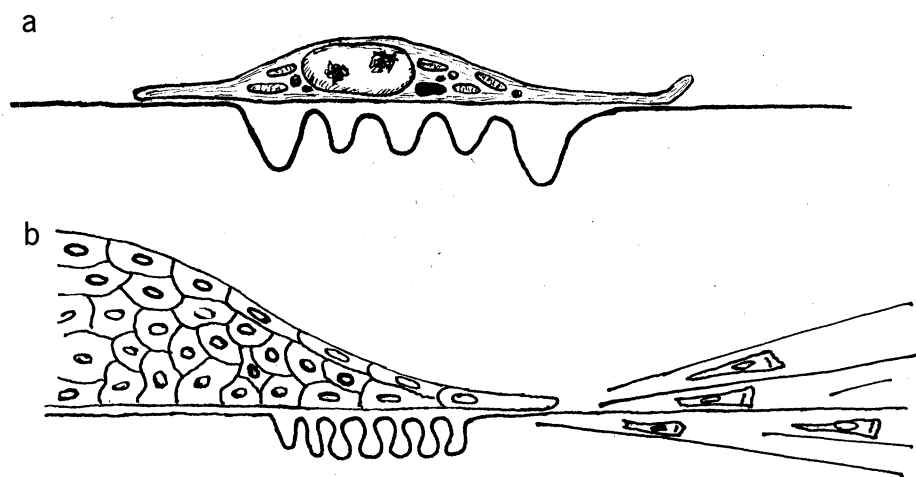


Fig. 1. (a) Diagrammatic side view of an individual cultured fibroblast distorting and wrinkling the elastic silicone substratum upon which it has spread and is crawling. (b) Diagrammatic side view of the margin of an explant whose cells are spreading outward on a silicone rubber substratum. The traction forces exerted by the outgrowing cells compress the rubber sheet beneath the explant and stretch it into long radial wrinkles in the surrounding area.

blasts, liver parenchyma cells, liver macrophages, pigmented retina cells, sensory and sympathetic neurons and glia, as well as cells of the line PTK-1. Of these, only macrophages and nerve fibers proved to be too weakly contractile to produce visible distortion of the elastic substrata. We have routinely cultured cells on these substrata for as much as several weeks continuously without any indications of toxicity to the cells or of alterations in the substrata, other than the physical distortions due to traction. We therefore judge that both the silicone fluid and rubber are completely nontoxic and inert, as their chemical nature and previous medical uses as implants would lead one to expect (9).

As they spread on these surfaces, the cells slowly pull the rubber sheet centripetally past their lower surface, stretching and distorting it enough to produce pronounced wrinkles in the rubber (Figs. 1a and 2, a to e). The additive effect of the traction exerted by many hundreds of cells crawling outward from explants produces a striking degree of distortion and wrinkling of these rubber substrata (Fig. 1b). Beneath and behind the outgrowth zone itself, the rubber is compressed into an accordion-like mass of circumferential folds, while the area of rubber surrounding this zone is stretched into many long radiating wrinkles oriented along the axes of the tension created. Within as little as 48 hours, fibroblast ex-

plants can pull in and fold up beneath themselves areas of rubber sheet a millimeter or more across. Yet even this degree of distortion represents true elastic stretching, rather than either plastic distortion or chemical shrinkage, since when the cells are detached with EDTA (ethylenediaminetetraacetic acid), the sheet quickly reexpands to its original shape (although this reexpansion is often partially retarded by adhesion between folds).

This pattern of substratum distortion around explants seems to be the same as that observed in plasma clots, even to the extent that peripheral cells become radially oriented by contact guidance along the radiating wrinkles in the rubber

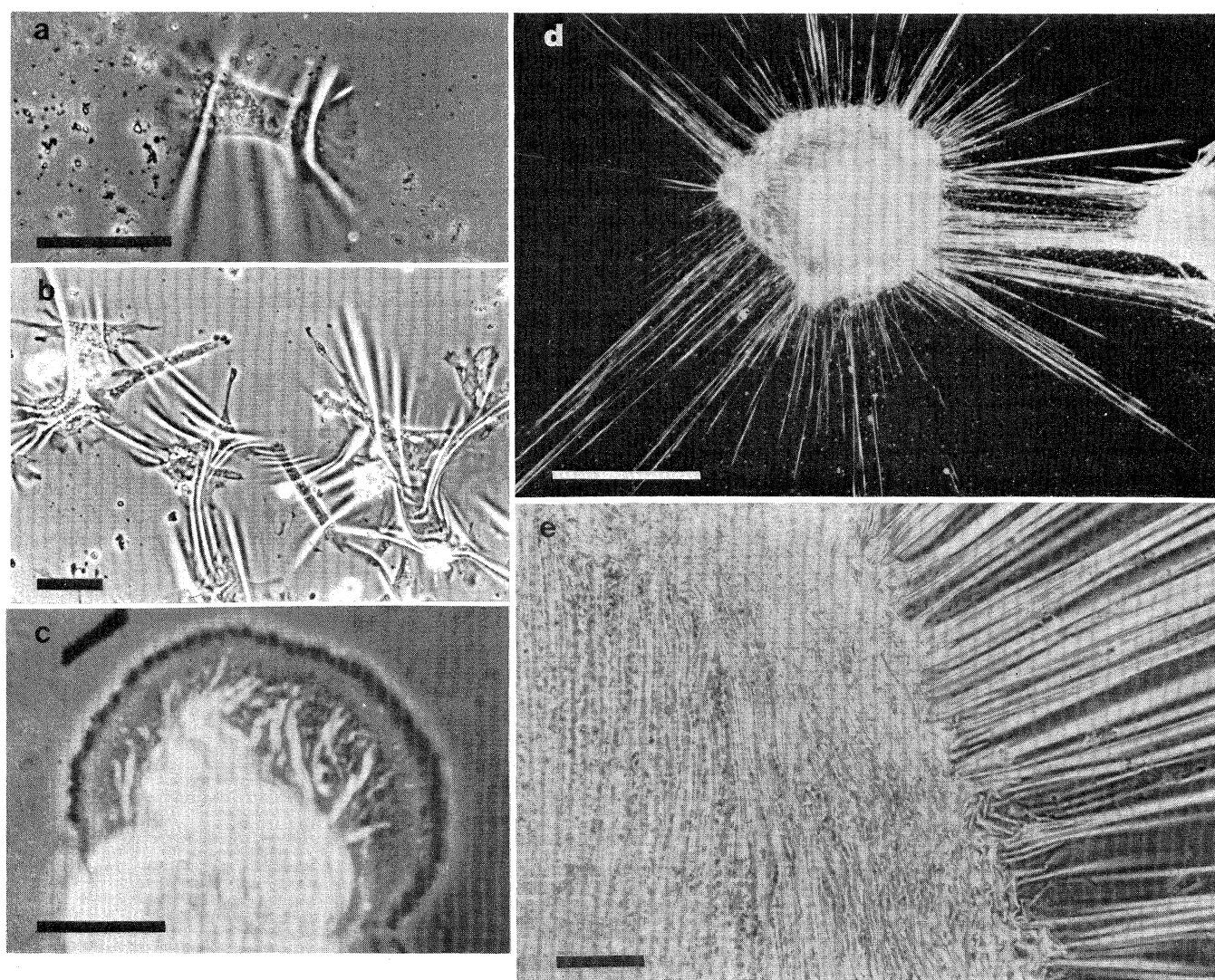


Fig. 2. (a) An individual chick heart fibroblast whose locomotion and contractility have visibly wrinkled the silicone rubber substratum upon which it is crawling (the bar is 50  $\mu$ m long). (b) Lower magnification view of about a dozen chick heart fibroblasts and the complex pattern of distortions produced in the rubber substratum by their locomotory traction (the bar is 100  $\mu$ m). (c) Higher magnification view of an individual PTK-1 cell just beginning to spread onto a silicone rubber sheet after trypsinization. Ruffling activity is seen as a dark band around the periphery, while the wrinkles being generated in the substratum are seen as irregular white bands beneath the cell body (from an individual frame of a 16-mm time-lapse film; the bar is 20  $\mu$ m long). (d) Very low magnification, dark-field illumination view of a chick heart explant that had been spreading on a silicone rubber substratum for 48 hours. The bright radiating lines are stress wrinkles. Notice the "two-center effect" of wrinkles running between the large central explant and the smaller one at the edge of the photograph at the right (the bar is 1 mm long). (e) Higher magnification, phase-contrast view of the marginal outgrowth zone of the same explant as shown in (d). The compression folds beneath the explant are seen on the left and the radial stress wrinkles around it are on the right (the bar is 100  $\mu$ m long).

surface. With two nearby explants one even observes Weiss's classic two-center effect (Fig. 2, d and e), yet there is no possibility that the rubber substratum is being dehydrated (as it was never hydrated) and no indication of any other biochemical alteration.

Thus we conclude that the apparent "shrinkage" of plasma clots and other distortable substrata by outgrowing tissue cells is caused primarily, if not entirely, by the traction forces cells exert in order to propel themselves outward. But this reinterpretation of Weiss's classical observations is not at all inconsistent with his larger theory that the stretching orientation of extracellular fiber networks could serve to guide cell migrations within the embryo (10). Our observations imply that cell locomotion in vivo could generate large tension fields, orienting extracellular fibers over considerable distances. It would be unlike evolution not to make use of these fields to guide morphogenesis.

From time-lapse films of the distortions produced in these rubber substrata by individual crawling cells it appears that tissue cells exert traction as a shearing force in the plane of the lower surface membrane. The effect is much as if the bottom of the cell were occupied by an invisible tractor tread of some kind. Using interference reflection microscopy (11), we observed that the fibroblasts' contacts with the rubber substratum are distributed in the same spatial pattern as one sees when they are cultured on glass (12). These contacts, corresponding to the adhesions through which the tractional shear forces are exerted, are concentrated just distal to the advancing margins, mostly in a band from 5 to 25  $\mu\text{m}$  rearward from this margin. This is also the region in which the compression wrinkles begin, implying that the forces are exerted beneath the cell, rather than along the advancing edge itself. Observations with a polarizing microscope revealed that the silicone rubber develops so little birefringence, even when highly stretched, that the cytoskeletal birefringence is not obscured by that of the substratum.

To measure the absolute magnitude of the shear forces exerted by individual fibroblasts we use glass microneedles whose flexibility is calibrated by hanging small paperweights on their ends. Then, using a deFonbrunne micromanipulator, the tips of these needles are pushed laterally upon the rubber until this surface is distorted to the same degree as it is by the cells. The shear force required is determined by observing the degree of lateral bending of the microneedle, and

comparing this with the bending produced by the weights. Initial measurements indicate that chick heart fibroblasts exert shear forces in excess of 0.001 dyne/ $\mu\text{m}$  of advancing margin.

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We dedicate this report to the late Michael Abercrombie whose powerful yet kindly intellect so greatly advanced the study of cell behavior. The original idea for using flexible culture substrata developed out of a lunchtime conversation with him, although he doubted it would work. The ideas of G. Dunn and A. Middleton were also most helpful.

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## "Transdifferentiation" of C6 Glial Cells in Culture

**Abstract.** *The activities of cyclic nucleotide phosphohydrolase, an enzyme marker for oligodendrocytes, and glutamine synthetase, an enzyme marker for astrocytes, were studied at early (21 to 26) and late (82 to 88) cell passages. The activity of cyclic nucleotide phosphohydrolase was markedly high and that of glutamine synthetase was low in the early passages, but this relation was reversed in the late passages. These findings suggest a "transdifferentiation" of C6 glial cells with passage in culture.*

Many investigators have used C6 glial cells, a rat glioma line, to study glial cell properties and function (1). The cell line has generally been designated as an astrocytoma (2). Silbert and Goldstein (3) reported that cells in the C6 line differentiated to cells resembling astroglia after treatment with amethopterin. In addition, a study by Bissel *et al.* (4) showed the induction of glial fibrillary acidic protein (GFA), an astrocyte marker, when the cells were maintained in sponge foam matrices. They suggested that C6 cells

represent an undifferentiated glial cell with the potential to differentiate into astrocytes. On the other hand, 2',3'-cyclic-nucleotide 3'-phosphohydrolase (CNP) (E.C.3.1.4.1), a marker for myelin and oligodendroglia, has been found in these cells (5). Likewise, the oligodendrocyte marker, glycerol phosphate dehydrogenase (GPDH) (E.C.1.1.1.8), is present and inducible in these cells (6).

The enzymes glutamine synthetase (GS) (E.C.6.3.1.2) and CNP have been associated with astrocytes and oligo-

Table 1. Changes in 2',3'-cyclic-nucleotide 3'-phosphohydrolase activity in C6 glial cells of different passages.

Cell passage	Day in culture	Plating cell density	Enzyme activity*		Picograms of protein per cell
			Units per milligram of protein	Units per 10 <sup>6</sup> cells	
26	4	0.25 × 10 <sup>6</sup>	0.269 ± 0.041†	0.050 ± 0.004	222 ± 35
		1.00 × 10 <sup>6</sup>	0.320 ± 0.044	0.078 ± 0.014	200 ± 4
	10	0.25 × 10 <sup>6</sup>	0.618 ± 0.048	0.119 ± 0.006	181 ± 3
		1.00 × 10 <sup>6</sup>	0.688 ± 0.032	0.134 ± 0.008	196 ± 14
88	4	0.25 × 10 <sup>6</sup>	0.219 ± 0.012	0.053 ± 0.005	242 ± 21
		1.00 × 10 <sup>6</sup>	0.260 ± 0.021	0.044 ± 0.011	170 ± 15
	10	0.25 × 10 <sup>6</sup>	0.203 ± 0.031	0.033 ± 0.005	186 ± 13
		1.00 × 10 <sup>6</sup>	0.125 ± 0.009	0.023 ± 0.002	186 ± 13

\*Units of activity represent micromoles of phosphate produced per minute, derived from a standard curve phosphate produced from 2'-adenosine monophosphate. †Mean ± standard error of the mean of four to five samples.