served also in *E. patella, E. daidaleos, E. plumipes, E. aediculatus*, and to a lesser extent in *E. eurystomus*. No such effect is found in either *E. woodruffi*, which is closely related to the above mentioned species [it has the same pattern of nine type 1 fronto-ventral cirri (*6*)], or in *E. crenosus* (only one stock tested so far) which belongs to the freshwater *Euplotes* species with ten fronto-ventral cirri.

Predator induced defenses have been reported for rotifers (*7, 8*), cladocerans (*3, 9*), and bryozoans (*4, 10*). Our example demonstrates the existence of this ecologically interesting feedback mechanism for protozoa. A chemical characterization of the signal substance that triggers the defensive response in the prey organism has so far been attempted only for the factor released by the carnivorous rotifer *Asplanchna brightwelli* (*8*). On the basis of his finding (*8*) that the activity of *Asplanchna*-conditioned medium was destroyed by treatment with Pronase, Gilbert suggested that the *Asplanchna*-factor is a protein (*8*). We assume that the *Lembadion*-factor which causes *Euplotes* to change its cell shape is also a polypeptide. That it was not found to be readily inactivated by Proteinase K may indicate an unusual amino acid composition. Further studies are, however, required before final conclusions can be drawn.

The possibility of inducing *Euplotes* to change its cell shape drastically by exposing it to a defined chemical signal may be useful for studies in developmental biology. Since the cells respond directly to the morphogen, the changes can be induced synchronously under controlled conditions in large numbers of cells. This should make it possible to discern the chain of events occurring from the binding of the morphogen to specific cell receptors and leading to the reconstruction of the cytoskeleton as a function of altered gene activity.

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Diagnostic Ultrasound and Sister Chromatid Exchanges: Failure to Reproduce Positive Findings

Abstract. *Human lymphocytes were exposed in vitro to ultrasound from two clinical devices, one of which was previously reported to have increased the frequency of sister chromatid exchanges. The ultrasonic exposures had no significant effect on the frequency of sister chromatid exchanges from three blood donors. Exposure to ultrasound also had no effect on cell cycle progression. A concomitant positive control (mitomycin C) resulted in a significant increase in sister chromatid exchanges.*

Whether the frequency of sister chromatid exchanges (SCE's) is increased as a result of exposure to diagnostic ultrasound has become an important issue since the initial positive report by Liebeskind *et al.* (*1*). In that study the frequencies of SCE's from lymphocytes in vitro exposed to ultrasound were higher than those from controls. This effect was not observed with comparably exposed HeLa cells (*2*). Subsequently, there have been three additional reports (*3, 4*) of increased frequencies of SCE's with exposure to ultrasound; two of the studies used human lymphocytes (*4*), the third used Chinese hamster ovary cells (*3*).

Many reports, however, have not indicated an increase in SCE's after exposure to ultrasound (*5–8*). Brulfert *et al.* (*5*) and Miller *et al.* (*7*) were unable to verify the positive results of Liebeskind *et al.* (*1*). Although the conditions for the three studies were generally similar, different ultrasound equipment was used. The next logical step, therefore, was to obtain the ultrasound exposures of lymphocyte cultures in vitro with the same clinical devices used by Liebeskind *et al.* (*1, 2*)—a Reflectoscope UM727 (Sperry Rand) and Ekoline-20 (Smith-Kline). We focused on the Liebeskind *et al.* study (*1*) because it was the first to report an increase in SCE frequency with diagnostic ultrasound exposures. We sought to verify their results with their equipment and cell culture facilities to elucidate the physical mechanism of action.

Two sets of experiments were conducted. The first used two ultrasound devices for exposures, and the cells were cultured in Ham's F10 medium with bromodeoxyuridine (BrdU) available continuously; in the second, we used McCoy's 5A medium, added BrdU 22 hours after the culture was initiated and exposed to ultrasound with the Reflectoscope unit.

Blood from three donors was drawn into syringes containing heparin and allowed to sediment for 2 to 3 hours at room temperature so that the leucocytes were separated by gravity from the erythrocytes; one of the donors had also participated in earlier experiments (*1*). For each donor, two 250-ml flasks (Falcon 3024) containing 28 ml Ham's F10 medium (or McCoy's 5A medium), 15 percent fetal bovine serum, phytohemagglutinin (Wellcome) (1 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), 2 m*M* L-glutamine, heparin (4 U/ml), and 9 μg/ml BrdU were seeded with 2 ml of plasma containing leucocytes. In the second set of experiments only 0.5 ml of plasma was added so that the cell concentration was 2×10^5 cell/ml (*1*). For one donor an extra flask was used, with mitomycin C (Sigma) added at a final concentration of 0.033 μg/ml [0.1 μg/ml (*1*)], to provide a positive control with frequent SCE's. Cells were grown in the dark at 37°C in a CO_2 incubator.

At 47 to 48 hours the cultures were exposed to ultrasound. The contents of the two flasks from each donor were mixed and the cultures transferred into three 15-ml plastic tubes. Each tube was completely filled with culture medium and then capped with a sterile sheet of Parafilm. Care was taken to exclude any bubbles under the Parafilm. The tubes were maintained in a water bath at 37°C in the dark; one tube was a control, the others were exposed with one of the ultrasound devices for 30 minutes. The mitomycin C control was also transferred to a 15-ml tube and maintained in

Table 1. Dosimetric determinations for the diagnostic ultrasound devices used.

Measure	Reflecto-scope (Sperry Rand)	Ekoline-20 (Smith-Kline)
Frequency	2.0 MHz	2.2 MHz
Transducer diameter	13 mm	16 mm
Intensity (SATA)	4 mW/cm^2	12 mW/cm^2
Peak intensity (SATP)	4 W/cm^2	49 W/cm^2
Beam width at 5 cm	1.3 cm	1.3 cm
Beam area	1 cm^2	1 cm^2
Pulse repetition rate	~700 per second	~200 per second
Pulse length	~2 μsec	~2 μsec

the water bath for the exposure duration. The cultures were then returned to 250-ml flasks, and 15 ml of fresh medium was added. The cells were harvested after 67 to 69 hours of culture, Colcemid was added to the medium 2 to 4 hours before harvest to a final concentration of 0.1 μg/ml. The cells were centrifuged at 1000 rpm for 5 minutes. They were then resuspended in 0.075 KCl for 8 to 10 minutes and fixed in three changes of 3:1 mixture of methanol and glacial acetic acid.

Cells were "dropped" onto clean microscope slides and allowed to dry. They were then stained in a Hoechst 33258 solution (5 μg/ml, *p*H 7) for 10 minutes, mounted in phosphate buffer, exposed to blacklight (GE F15T8 BLB, 15 W) for about 8 to 10 minutes at a distance of 2 cm, and then stained with a 4 percent Giemsa solution at *p*H 7 for 10 minutes (*9*).

The slides were coded and scored without knowledge of regimen. Only cells showing 46 chromosomes were analyzed; 50 to 230 metaphases were scored for each regimen per experiment. The slides were decoded at the conclusion of the scoring, and the data were analyzed by *t*-tests. The slides of cells incubated with mitomycin C were included in the scoring.

For the first set of experiments, 30 coded slides from each of two donors were scored by two of us (W.F.M. and D.J.-K.). Their values for SCE frequencies were returned, decoded, and included in the results of this investigation. In addition the slides were also analyzed for the percentages of first-, second-, and third-division mitotic cells (*10*) according to an analysis of variance with an experimental blocking factor and a treatment factor, both having two levels.

Specific dosimetric determinations for each ultrasound device are summarized in Table 1. The dosimetric analyses indicated that the exposures in the present experiments were comparable to those used by Liebeskind *et al.* (*1, 2*). Aquasonic transmission gel was used as a coupling agent. The transducers were coaxially aimed down into 17 × 100 mm sterile polypropylene test tubes (Falcon 2006) that were completely filled with the cell suspensions and covered with a 12-μm thickness of sterile Parafilm.

The cell suspensions were exposed to ultrasound in a 37°C water bath (or sham exposed) for 30 minutes in the dark, after which they were placed in 250-ml culture flasks and returned to incubators.

The results of experiment 1 are summarized in Table 2. For each set of data a *t*-test was used to compare frequencies of SCE's per cell for control and exposed cells. In none of the 14 comparisons was there a significant difference ($P > 0.05$) between exposed and control SCE frequencies. There were, however, noticeable differences among the scorers for SCE's; scorer 1 had consistently lower SCE scores than scorer 2 or 3. There was no consistent difference between scorers 2 and 3. SCE frequency for cells exposed to mitomycin C was significantly greater than that for controls ($P < 0.001$). The results of data collected for the percentage of first-, second-, and third-division cells are summarized in Table 3. Analysis of variance indicated no significant differences ($P > 0.05$) resulting from treatment factors; this result is similar to that reported by Miller *et al.* (*7*).

The results of experiment 2 are summarized in Table 4. There were no significant differences ($P > 0.05$) in any of the three exposed and control SCE frequencies, but a fourfold increase in SCE frequency of cells exposed to mitomycin C.

Our negative results concerning SCE frequencies of in vitro human lymphocytes exposed to the ultrasonic field of the Ekoline-20 clinical device are consistent with those reported for similarly

Table 2. Sister chromatid exchanges per human lymphocyte exposed to ultrasound; S.E.M., standard error of the mean.

Donor and condition	Scorer 1		Scorer 2		Scorer 3	
	*n**	Mean ± S.E.M.	*n**	Mean ± S.E.M.	*n**	Mean ± S.E.M.
V.C.						
Control	50	12.90 ± 0.65	100	16.43 ± 0.46	200	15.51 ± 0.38
Reflectoscope	50	11.92 ± 0.64	100	15.25 ± 0.42	200	15.45 ± 0.54
Ekoline	50	11.32 ± 0.49	100	15.30 ± 0.44	200	15.56 ± 0.22
A.B.						
Control	50	10.92 ± 0.53	100	14.89 ± 0.41	230	14.56 ± 0.39
Reflectoscope	50	11.38 ± 0.46	100	13.80 ± 0.38	230	14.87 ± 0.48
Ekoline	50	11.92 ± 0.55	100	14.12 ± 0.40	230	14.64 ± 0.37
R.B.						
Control	50	8.22 ± 0.35				
Reflectoscope	50	9.04 ± 0.44				
Ekoline	50	8.24 ± 0.49				
Mitomycin C	25	44.92 ± 2.00				

*Number of second-division metaphase cells analyzed.

Table 3. Distribution (average percentage ± S.E.M.) of first, second, and third mitotic figures in lymphocytes exposed to ultrasound.

Donor and condition	Mitotic figure		
	First	Second	Third
V.C.			
Control	42.31 ± 1.98	44.79 ± 2.13	12.90 ± 0.83
Reflectoscope	42.69 ± 1.67	46.19 ± 1.36	11.13 ± 0.88
Ekoline	44.36 ± 1.94	43.57 ± 1.46	12.41 ± 1.20
A.B.			
Control	35.51 ± 1.22	39.67 ± 1.59	24.82 ± 1.45
Reflectoscope	25.77 ± 2.00	45.59 ± 3.27	28.64 ± 2.29
Ekoline	31.70 ± 2.84	40.85 ± 1.78	27.46 ± 2.20

Table 4. Sister chromatid exchanges in human lymphocytes exposed to the Reflectoscope unit.

Donor and condition	SCE's per cell (mean ± S.E.M.)
V.C.	
Control	11.54 ± 0.53
Reflectoscope	12.24 ± 0.56
R.B.	
Control	10.36 ± 0.55
Reflectoscope	10.44 ± 0.47
P.G.	
Control	9.46 ± 0.47
Reflectoscope exposed	9.70 ± 0.49
Mitomycin C	>40

exposed HeLa cells in vitro (*2*). Our Reflectoscope results, however, are inconsistent with those reported for similarly treated human lymphocytes in vitro (*1*). We have no explanation for the discrepancy. In both cases, however, the positive control (mitomycin C) yielded significantly more SCE's.

In an initial attempt to verify the positive results of Liebeskind *et al.* (*1*), in vitro whole blood was exposed to ultrasound under conditions similar to theirs, but our results were negative (*7*). To better approximate their exposure conditions, lymphocytes obtained by gravity sedimentation were exposed to intensities of ultrasound that spanned those used by Liebeskind *et al.* (*1*). The results were again negative (*5*). Experiments were then conducted with continuous-wave ultrasound from an experimental unit under conditions that induced a small but significant amount of cell destruction: among the surviving cells there was no increase in the frequency of SCE's.

In experiment 1, the same ultrasound devices and cell culture facilities as used earlier (*1, 2*) were used, as were the same general procedures (for example, Parafilm cover, blood composition, one common donor, exposure intensities), yet the results were negative. Experiment 2 included the same culture medium (McCoy's 5A) used by Liebeskind *et al.* (*1*), as well as the same time of BrdU addition, and the results were also negative.

The positive results of Liebeskind *et al.* (*1*) have not been verified. We conclude that the reasons for their obtaining such results may be coincident with some subtle yet unidentified procedural factor.

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A Renal Countercurrent System in Marine Elasmobranch Fish: A Computer-Assisted Reconstruction

Abstract. *Computer-aided techniques were used to reconstruct the complex renal tubular system in the dorsal kidney region of a marine elasmobranch fish, the little skate (*Raja erinacea*), from a series of light micrographs of serial sections. It was established that five individual segments of one nephron, consisting of two loops and a distal tubule, are arranged in parallel within an elongated closed tissue sac. Capillaries, which form a network around these nephron segments, enter and exit this sac at the same end. This anatomical arrangement suggests that a complex renal countercurrent multiplier system may be important in fluid regulation in these fish.*

Typically, marine fish are faced with the problem of dehydration due to the high osmolality of the surrounding seawater (*1*). Marine elasmobranch fish appear to resolve the problem by maintaining high urea concentrations in plasma and tissue, thereby elevating the osmolality of their internal milieu to nearly that of the surrounding seawater (*2*). An extremely small fraction of urea is excreted (*3*), and studies of the kidney have been done to determine how this urea conservation is achieved (*1–5*). However, the extremely complex configuration of the elasmobranch nephron has impeded physiological studies of the anatomical site of urea reabsorption and of some of the cellular mechanisms that are involved (*6*). This nephron complexity is evidenced by the fact that elasmobranchs are members of the only vertebrate class (Chondrichthyes) in which the nephron configuration and epithelial sequence along its length to the collecting duct are not known (*6, 7*).

We used computer graphics to study nephron configuration. A three-dimensional reconstruction of the tubule in the complex dorsal region of a marine elasmobranch kidney shows that parallel tubular segments from a single nephron are tightly wrapped in a cellular sheath that also encloses a capillary network around the tubules. This anatomical arrangement presumably has the potential of facilitating a physiological countercurrent system.

The kidneys of little skates (*Raja erinacea*) and spiny dogfish (*Squalus acanthias*) were fixed with buffered glutaraldehyde by vascular perfusion (*8*). We used 118 light micrographs of serial sections from the skate to outline and sequence 2945 tubule contours (outer circumference of tubules) of the sectioned nephron as it completed its course within the dorsal region of the kidney. The same photographs were used to outline the cellular peritubular sheath that surrounds bundles of these tubule segments (*9*). Because a complex computer-generated image was expected, the profiles of the blood vessels accompanying the tubules were not entered into the computer. The course of these vessels was simpler than that of the adjacent tubules and it was traced in the same serial sections by light microscopy.

The architectural complexity of nephrons in the dorsal region was greater than what could be reconstructed by techniques such as wax modeling. For the computer-assisted reconstruction, we used a hierarchical data-base design that accommodates the complex branching nature of biological structures and performs coordinate transformations necessary to convert serially sectioned biological materials into three-dimensional display coordinates. Solid modeling algorithms were used to generate the video images (*10, 11*).

Beginning with the urinary pole of the renal corpuscle, we traced the course of the nephron as it first entered the bundle zone in the dorsal region of the kidney