WSN

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TECHNICAL INFORMATION BULLETIN

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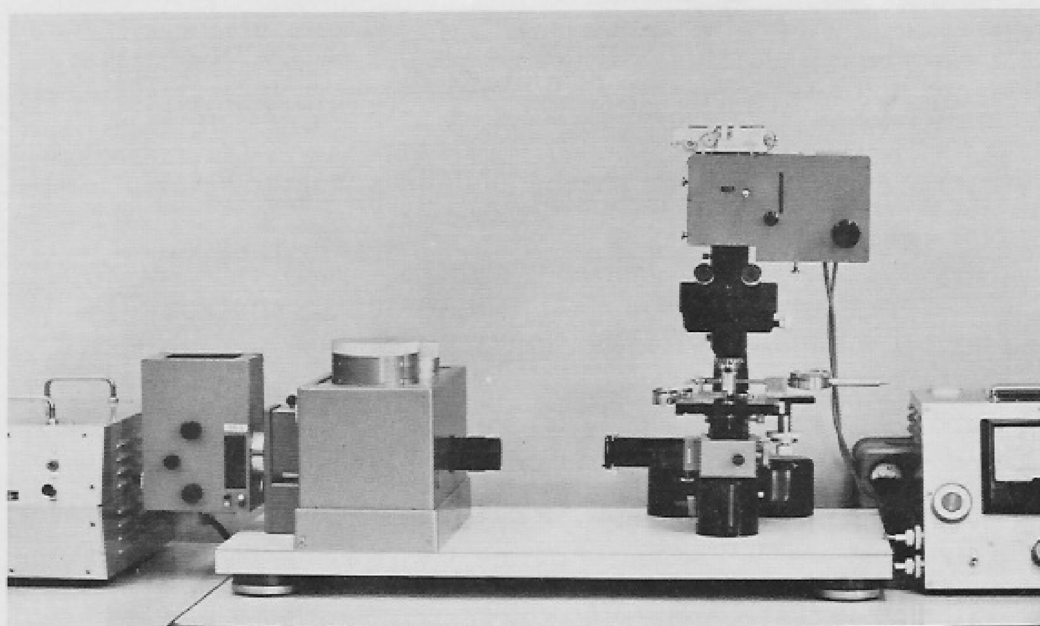
QUANTITATIVE MICROSCOPY

The microphotometer has been designed for ease and convenience of operation. The observer can remain seated, locate the measuring area in the binocular observation tube and position it with great accuracy under binocular observation. A slight turn of the head only is required to check the position on the viewing telescope and to make final corrections. All controls are arranged so that the arms can be rested on the table. The photomultiplier is prealigned for linearity, and it is not possible to accidentally disalign it. The microphotometer fits all FSA tubes.



Two oculars are permanently built into the unit, a GF 10 x and a GF 16 x. These wide-field oculars are mounted on a slider. It is easily possible to exchange them for other oculars, such as for example oculars with focusable eyelenses which accept reticules. In this case rectangular stops, or stops of any geometric shape can easily be introduced at the proper optically conjugated plane. Above the ocular a 1/3 reducing objective is mounted. This objective serves two purposes: It projects the image onto the filmplane of the 35mm camera mounted on top, and also via a slider-mounted 90° prism, into the plane of a stop limiting the measuring field.

The presence of the 1/3 reducing objective keeps the objectives - and particularly high-dry systems - at the calculated working distance and thus avoids spherical aberrations. These would appear as severe image haze and not only introduce measuring errors, but also cause poor image quality in the viewing telescope.

Above the 1/3 reducing objective a Compur-shutter is mounted, followed by the already mentioned 90° prism. This prism is mounted on a slider, so it can readily and with great precision be moved in and out for photography or measurement.

The left compartment of the microphotometer is separated from the right side, which houses the photomultiplier by means of a light tight wall with only a small opening. This is located directly in front of a subassembly carrying a rotatable disc with precision-drilled fixed paertures. These have been drilled to appear in absolutely parcentric positions, and these positions in turn have again been made very accurately parcentric with the adjustable iris diaphragm.

The diameters of the fixed apertures are 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mm, from which one can easily compute the field diameter as follows:

M objective x 1.25 tube factor x M ocular x 1/3 = total magnification.

$$\frac{\text{Hole diameter in mm x 1000}}{\text{total magnification}} = \text{measuring field diameter in abject in } \mu$$

Numerical example: 90 x 1.25 x 16 x 1/3 = 600 total magnification

$$\frac{0.5 \times 1000}{600} = \frac{500}{600} = 0.83 \mu$$

For viewing the light is deflected by a 30° prism into a 50 mm objective, and via another 90° prism into a viewing telescope. For measurement the 30° prism is pulled down and the light reaches the photocathode of the photomultiplier directly. The photomultiplier is pre-aligned so that complete linearity exists over the range of practical measuring fields. The ocular in the viewing telescope is a 10 x measuring ocular with a reticule. The viewing telescope as such is set into a centering mount so that precise parcentricity between binocular tube and viewing telescope can be achieved.

MICROINTERFEROMETRIC MEASUREMENTS

Interference microscopes are highly accurate instruments for the measurement of refractive index and geometric thickness in microscopic specimen. In medical and biological research, however, the interference microscope is primarily used as an ultrasensitive balance. The dry mass of cells or cell components can be weighed optically with an accuracy of 10^{-14} grams.

The applications include direct dry mass determinations as well as measurements of changes or differences of dry mass, differential dry mass determinations before and after treatment with various enzymes, and measurements of nucleic acid metabolism. Equally important are dry mass measurements in conjunction with other quantitative micromethods. In microspectrophotometry the interference microscope can be used to relate stain intensity or extinction to the dry mass of the stained substrate and thus to establish the stoichiometry of a histochemical color reaction. This can be done by measuring the difference in dry mass before and after a suitable chemical or enzyme treatment. The difference in extinction is then referred to the difference in dry mass which has been measured in absolute units. In this procedure one does not have to assume complete removal of a substance by enzyme treatment.

An elegant method to study the nucleic acid metabolism of cell nuclei is the use of tritium labeled metabolites. The turnover is determined from a count of silver grains in the overlaying thin autoradiograph emulsion. It has been shown, however, that the penetration of tritium radiation is so extremely short that the self-absorption effect can under no circumstances be neglected. To obtain the correction factor the dry mass of the tissue components must be known, and this again is done by micro-interferometry.

The Mach-Zehnder circuit in the Leitz interference microscope permits measurements in extended tissue sections. It is not necessary to have free background in the field of view.

The practical measuring procedure is not complicated. Two consecutive photomicrographs are taken of the specimen, one with a fringe field, the other in interference contrast. The first negative serves to calibrate the galvanometer scale of a densitometric set-up directly in picogram dry mass / μ^2. The second exposure is used to take as many spot measurements in the specimen as one desires. It is not necessary to use an elaborate densitomater. A low power microscope with a light measuring device attached to the phototube is very satisfactory. Experience has shown that with this technique one can take approximately 300 measurements of dry mass / μ^2 in two days. The rather widespread belief that dry mass determinations require an undue amount of time and effort is simply not correct.

A practical example of a relative mass determination is shown in fig. 1. The chromosomes were photographed in interference contrast, and the negative scanned in an Ortholux densitometer. To help orientation the negative was enlarged by means of a Prado microprojector to an exact magnification of 1 : 10000. This, of course, by far exceeds the useful magnification, but offers the advantage that 1 cm^2 on the graph paper is equal to $1 \mu^2$. This makes integration and total dry mass determination easier. Also the densitometer has a much higher differential sensitivity to intensity differences than the human eye, so that the classical considerations with respect to resolution do not apply anyhow. The photomicrograph in fig. 1 was taken with a 50 x high dry objective. In fig. 2 an example is given where the dry mass scale was calibrated in absolute units. The specimen is a Purkinje cell in a thin section of rat cerebellum. Although the absolute calibration of the dry mass / μ^2-scale does not present any difficulty it should be pointed out that a measurement of the effects of enzyme treatment does not even require the absolute calibration, but only the comparison of the relative density of, for example, nucleolus and nucleus. Mass differences may, of course, be read off the relative mass scale as shown in fig. 1 in absolute units.

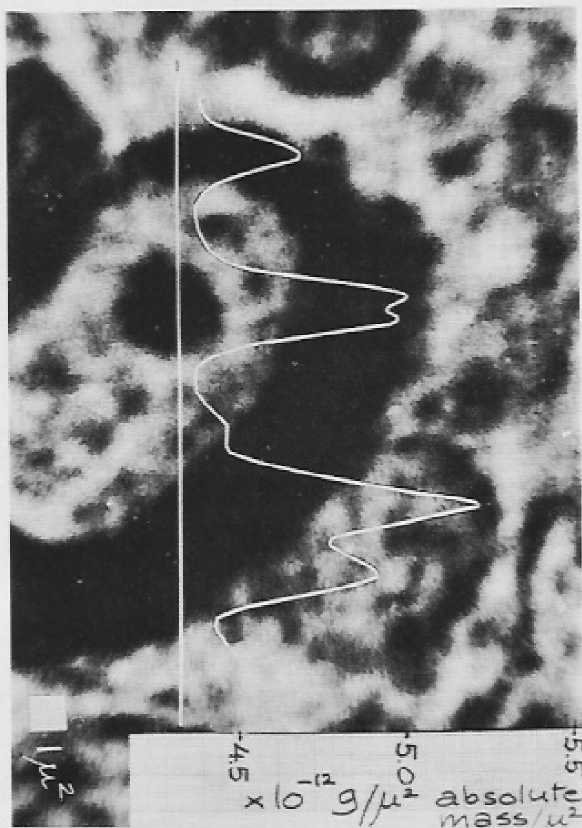


Fig. 1

A step-by-step description for the practical procedure is available upon request.

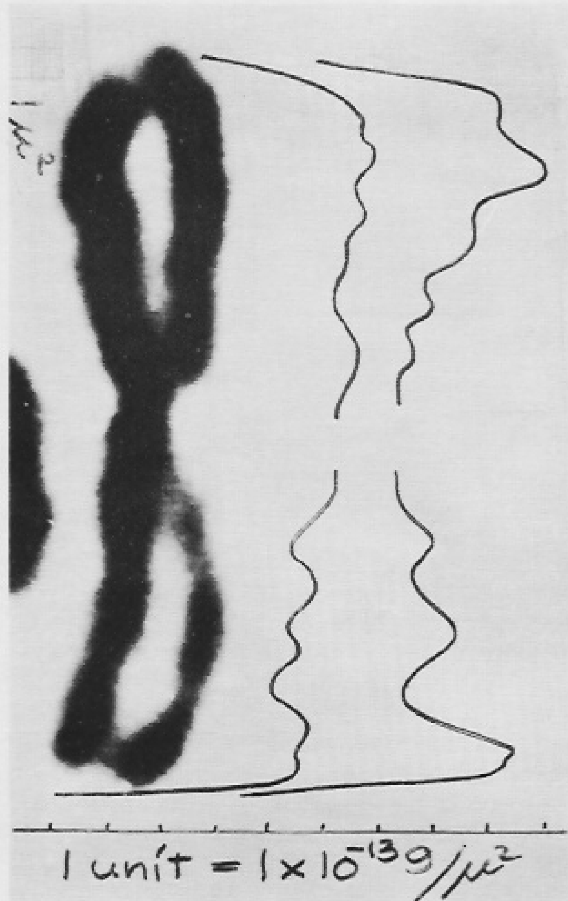


Fig. 2

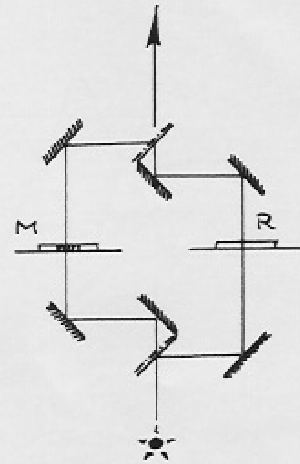


Fig. 3

In the following a short explanation of the measuring principle in the interference microscopy will be given.

The light in a typical interference microscope is divided into two partial beams. The "measuring beam M" passes through the specimen, which affects it in a characteristic manner. The "reference beam R" by-passes the object. This is shown in fig. 3. The first beam splitter in fact separates the light from each elementary emission act into the two partial beams: The wavefield of the measuring beam and that of the reference beam are therefore "coherent," which means that the two wavefields maintain constant relative positions, or phase relationships.

After the light in the measuring beam has passed the specimen, and the light in the reference beam a suitable blank, it is reunited. Because the two wavefields are coherent, they will mutually interact, or "interfere."

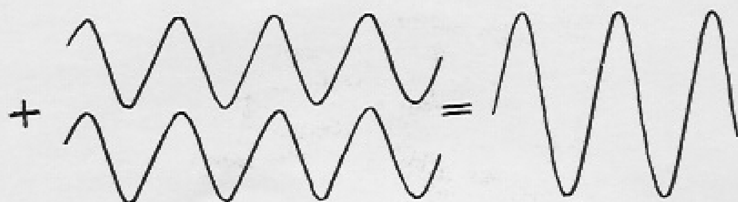


Fig. 4a

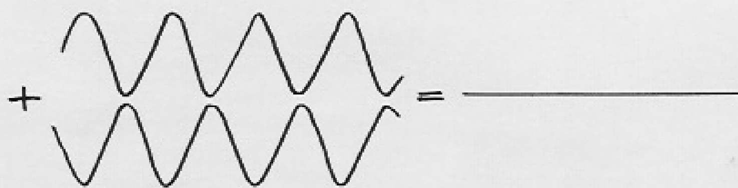


Fig. 4b

Whereever the relative positions of the waves in measuring and reference beam are such that wavecrest falls upon wavecrest, " constructive interference" will take place. The amplitudes of the two interfering waves will add up. But where the relative position between the measuring beam wavefield and that of the reference beam is such that a wavecrest in one coincides with a wavetrough in the other, "destructive interference" will occur, and the two waves will cancel each other out, provided they had equal amplitudes, see figs. 4a and 4b. The superposition of the two coherent wavefields of measuring and reference beam is shown in fig. 5. The straight lines symbolize the wavefronts and are assumed to indicate wavecrests.

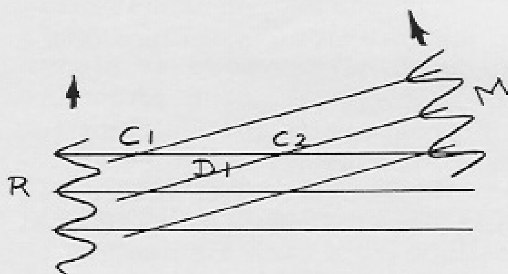


Fig. 5

R designates the wavefield of the reference beam, M that of the measuring beam. The angle of intersection is grossly exaggerated. It is also assumed that no specimen is placed in the measuring beam so that wavefronts remained straight and undisturbed.

At C1 two wavecrests of R and M coincide. Here constructive interference must take place, and a bright fringe will result. At D1 the wavecrest of R is opposed by the wavetrough of M. Destructive interference will take place and a dark fringe will be visible. At C2 the same conditions for constructive interference exists as at C1. The field of view of an interference microscope will therefore even when there is no specimen on the stage show a system of alternating bright and dark interference fringes.

The optic path difference from one dark fringe to the next, or from a bright fringe to the next bright fringe is exactly equal to one wavelength of monochromatic light (Hg 546 mμ), as can easily be seen from fig. 5. The distance from the maximum of a bright fringe to the center of the dark fringe is equal to an optic path difference of one half wavelength (Hg 546 mμ : 1/2 x 546 mμ = 273 mμ).

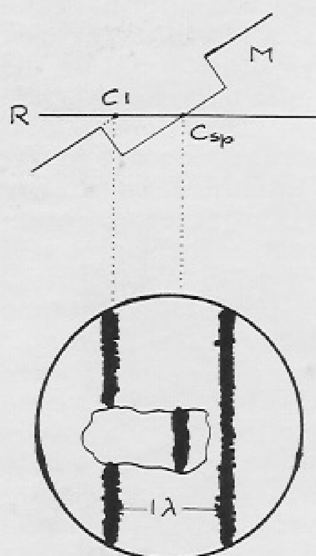


Fig. 6

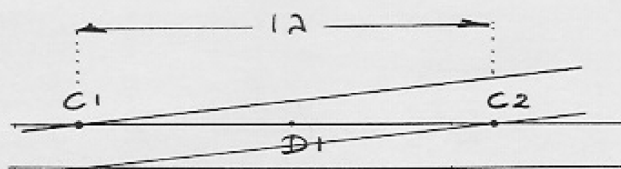


Fig. 7

A specimen with an optical density higher than the surroundings will delay that portion of the wavefront in the measuring beam which passes through it. This is shown in fig. 6.

One would expect a bright fringe at Cl, and that is where it is found in the empty field around the specimen. But the wavefront distorted by the optic path introduced by the specimen finds conditions for constructive interference only at CSp. An axially extending optic path difference appears as lateral fringe displacement.

Interference microscopes have a built-in calibration for optic path: the fringe distance in the undisturbed field. The displacement caused by the specimen can be read directly as a fraction or multiple of a wavelength. It is easy to see that a greater reading accuracy can be achieved when the scale is expanded,or the fringe distance increased. This can simply be done by reducing the angle of interaction between measuring and reference beam as shown in fig. 7.

One will not see a sharp bright fringe at Cl and a sharp dark fringe at Dl. The image intensity in the fringe pattern will gradually decrease from Cl to Dl.

At Cl conditions for exact constructive interference exist. The two interfering wavefields have no phase difference. At Dl the two wavefields have a phase difference of exactly 180°, or 1/2 wavelength. The image intensity at this point is zero. At Cl the amplitude is doubled.

Since intensity is defined as /amplitude/^{2} the image intensity at Cl is increased by a factor of four. Any phase difference between the two interfering wavefields greater than 0, and smaller than 180°, or half a cycle, or half a wavelength will lead to an image intensity between 4 times the original and zero. This means that a phase difference can be measured as a change in image intensity. Each point on the fringe contour between Cl and Dl indicates by its intensity exactly the phase difference relative to Cl, as seen in fig. 8. This principle of determining a phase difference by measuring the resulting image intensity is used in the most sensitive setting of an interference microscope, when it is adjusted for interference contrast. The planes of the wavefronts are made absolutely parallel, so that the fringe distance has become infinite. The field will now appear in an intensity determined by the phase difference between the two interfering wavefields. When this is zero the whole field will uniformly appear in maximum brightness. When the phase difference is smaller the field is less bright. When it becomes $\lambda/2$ the whole field will be completely dark.

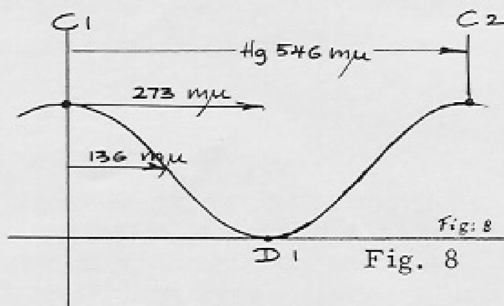


Fig. 8

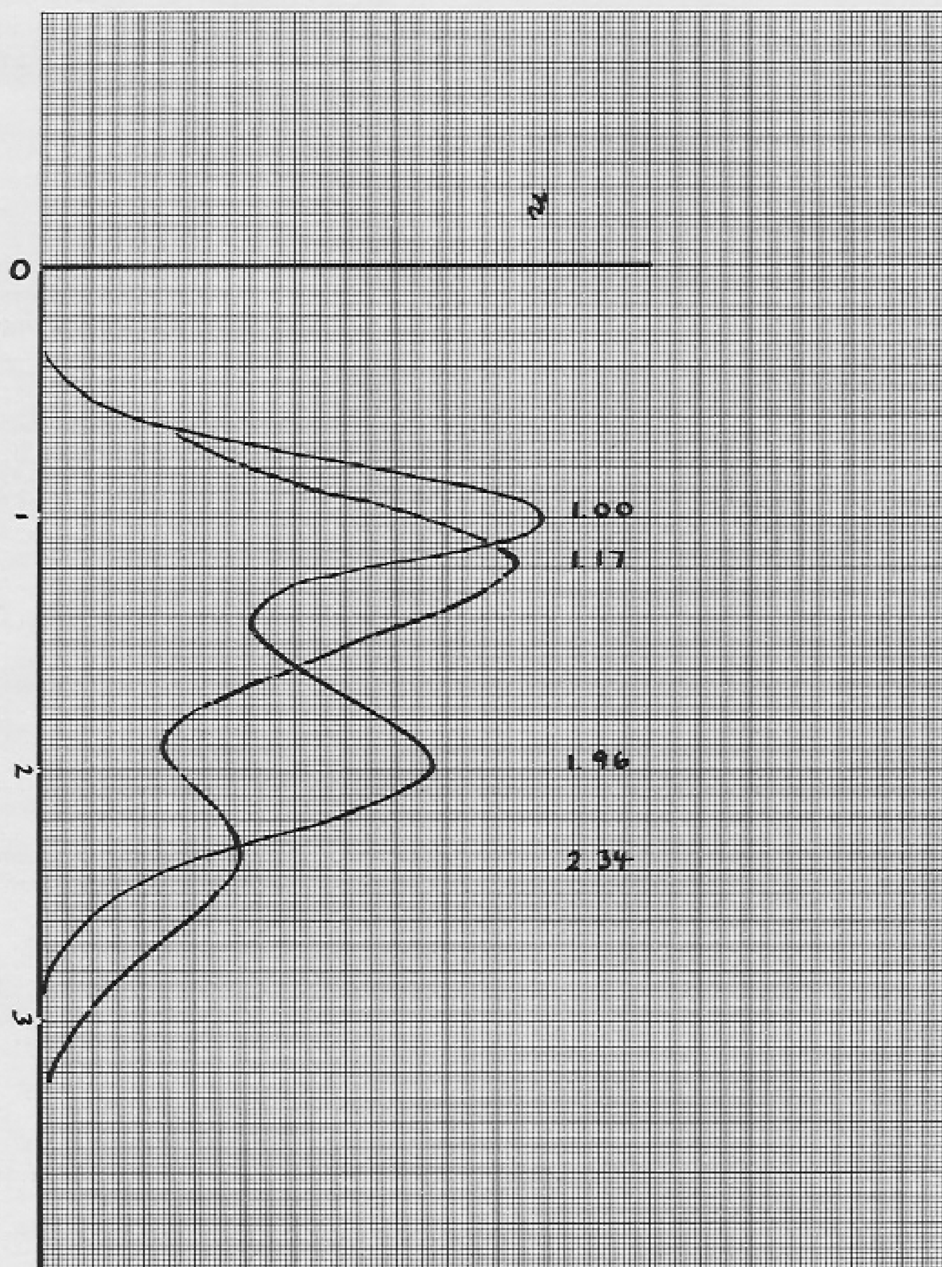
These conditions cannot, of course, simultaneously be fulfilled also for such portions of a wavefront in the measuring beam which have passed through a specimen. They will necessarily find different conditions for interference with the wavefield of the reference beam. The specimen area will therefore appear in a different image brightness. This brightness is a direct measure for the path difference introduced by the specimen. <u>An axially extending difference in optic path is quantitatively converted into a difference in image intensity.</u> The photomicrographs in fig. 1 and fig. 2 are quantitative images. The negative transparency at each point is a quantitative measure for the optic path difference introduced by the specimen. The optic path difference is directly proportional to the dry mass.

The principle of the practical measurement is therefore rather straight forward. The instrument is set to a fringe pattern and a black- and white photomicrograph is taken. Scanning the fringe contour will provide a calibration for the transparency of the negative as a function of optic path difference. The instrument is then set to interference contrast and the preparation is photographed again. The first negative serves to calibrate the galvanometer of a densitometric device directly in optic path difference and relative dry mass. The second negative serves to take as many point measurements of dry mass/μ^2 as one wants.

Many histochemical reactions can be measured quantitatively by the use of a microspectrophotometer.

The problem here was to find the relative amount of DNA present in the nuclei of a population of cells, and then to attempt to measure changes in this relative distribution as they may be caused by external influences such as radiation, pharmaceuticals, biochemicals.

The stain usually is the Feulgen stain, the measuring technique the 2-wavelength method.



A minimum of 150 nuclei should be measured for each distribution function. It is necessary to measure the absorption spectrum first on a homogenously stained region on each slide, to determine for which wavelength the exact extinction ratio of 2:1 is attained. This varies somewhat between preparations. Measurement in an area of granular distribution of absorbing matter would lead to a "diluted" spectrum.

The two wavelengths chosen here were 540 mμ and 500 mμ. Fig. 1 shows the distribution function found in two cell populations with an expected DNA-contents ratio of 2:1. The two maxima in each distribution curve actually occur on the position 1 and 1. 97, and 1. 17, and 2. 34 on the relative scale.

Both slides were stained in the same manner, and the observable shifts in the distribution curves are the result of the pretreatment which one cell population was subjected to.

One can see that the basic 2:1 ratio of DNA contents remains unchanged, but that the treatment affects the cells with the double DNA contents differently. One has to be careful, however, in concluding that the amount of DNA has changed: the treatment may merely have either masked or made available reaction sites and thus caused a change in staining density which affects the cells with twice the DNA contents more than the ones with the normal amount. When this possibility can be ruled out, the method permits a statistical analysis of small relative changes in DNA contents in a cell population as they might experimentally be produced.

THERMO COUPLE FOR HEATING STAGES

We have been asked for technical details regarding thermo couples as supplied with Leitz heating stages.

1) Thermo couple for heating stages 1350° centigrade
   One arm of this thermo couple is made of pure platinum, the other of platinum-rhodium. The percentage of rhodium amounts to 10%.

2) Thermo couples for heating stages 1750° centigrade
   This thermo couple consists of one arm with pure platinumand the other with an alloy of platinum-rhodium. The rhodium component is 13%. For temperatures above 1600° we use a thermo couple Pt-Rh 18.
   The rhodium content on one side is 6% and on the other side 30%, the rest is platinum.
   The thermo couples millivolt readings can be found on the table below.

Temperature - mV values for Temperature steps of 10° C

°C	0	10	20	30	40	50	60	70	80	90	100
0	0,00	– 0,002	– 0,003	– 0,002	– 0,001	+ 0,002	0,006	0,011	0,017	0,024	0,033
100	0,033	0,042	0,053	0,065	0,077	0,091	0,106	0,123	0,140	0,158	0,177
200	0,177	0,198	0,219	0,242	0,266	0,291	0,316	0,343	0,371	0,400	0,430
300	0,430	0,461	0,494	0,527	0,561	0,597	0,633	0,670	0,709	0,748	0,789
400	0,789	0,830	0,873	0,916	0,961	1,006	1,052	1,100	1,148	1,197	1,247
500	1,247	1,297	1,349	1,402	1,455	1,510	1,565	1,621	1,679	1,737	1,795
600	1,795	1,855	1,916	1,978	2,040	2,103	2,167	2,232	2,298	2,365	2,433
700	2,433	2,502	2,571	2,642	2,713	2,785	2,858	2,932	3,007	3,082	3,159
800	3,159	3,236	3,314	3,393	3,472	3,552	3,633	3,715	3,798	3,881	3,966
900	3,966	4,050	4,136	4,222	4,309	4,397	4,486	4,575	4,665	4,755	4,847
1 000	4,847	4,939	5,031	5,124	5,218	5,313	5,408	5,504	5,600	5,697	5,795
1 100	5,795	5,893	5,992	6,092	6,192	6,293	6,395	6,497	6,600	6,703	6,806
1 200	6,806	6,911	7,015	7,121	7,227	7,333	7,440	7,547	7,654	7,762	7,871
1 300	7,871	7,980	8,089	8,199	8,309	8,419	8,530	8,642	8,753	8,865	8,977
1 400	8,977	9,089	9,202	9,315	9,428	9,542	9,656	9,770	9,884	9,998	10,113
1 500	10,113	10,227	10,342	10,458	10,573	10,688	10,804	10,919	11,035	11,151	11,267
1 600	11,267	11,383	11,499	11,615	11,731	11,847	11,963	12,079	12,195	12,311	12,428
1 700	12,428	12,544	12,659	12,775	12,891	13,007	13,122	13,238	13,353	13,468	13,583

TIMER FOR ORTHOMAT CAMERA

We have had occasion to try out two timers manufactured by Industrial Timer Corporation, U.S. Highway 287, Parsippany, N.J. Our Micro-Laboratory reports that the following models would be of interest:

1) PABR 3M   for intervals from 3 seconds to 3 minutes

2) PABR 3H   for intervals from 3 minutes to 3 hours.

For prices and delivery information we suggest that you contact the above mentioned firm.

Pearlite, Magnification: 1000 x
Taken with MM 5 / Apo Plano 160 x N.A. 1.40

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