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Histophotometry: A New Method for Automated Histological Examination of Solid Tissue Samples Demonstrated on Bladder Cancer

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Summary. The purpose of this study was to demonstrate the ability of a new method to discriminate between benign and malignant tissue. Tissue samples from three patients with bladder tumours and one patient with bladder diverticula have been examined by histophotometry. This method, based on the correlation of nuclear volume and nucleic acid contents per cell uses thick formalin fixed samples. The results are obtained by measurements averaging more than one hundred thousand cells per sample. By this histophotometric examination a clear differentiation between malignant and benign tissue could be achieved. It was also possible to determine the degree of malignancy and to differentiate between infiltrating and noninfiltrating tumour, as well as inflamed bladder tissue. The results obtained histophotometrically were compared with the histological findings.

Key words: Bladder tumours, Histophotometry, Histology.

Since the basic work of Casperson (1), who developed U.V. spectrocytophotometry, it has been possible to describe cellular alterations not only morphologically, but also quantitatively. The application of the spectrophotometric method to the study of cancer tissue revealed that tumours are composed of cells which display differences in chemical composition and contents. A modified spectrophotometric method employs the visible absorption technique (8, 9). By using micro-absorption analysis it may be shown that DNA values are often increased in cancer cells (6). Both spectrophotometric methods yielded information which suggests that the larger volume of the nucleus of the cancer cell is associated with an increased DNA content, or with a more intense synthesis of DNA (4). The finding, that malignant cells have an enlarged nucleus has been confirmed by several investigators (4).

A correlation between DNA content and nuclear volume has been established (3). Alteration of that correlation is the first measurable indicator of the altered cell function (3).

All investigations using photometric techniques have been carried out on histological sections or exfoliated cells. A new method has been developed to determine the correlation between nuclear volume and nucleic acid content in solid, unprepared tissue samples. The first results for bladder tissues are the subject of this paper.

METHODS

For the measurements an approximately 1 mm thick slice of tissue is squeezed between 2 plane-parallel glass slides. The thickness of the tissue slice is not critical, and can range from 0.9 to 1.8 mm. The sandwich produced is mounted in the center of the goniometer (Fig. 1) and irradiated with a monochromatic UV-beam having a wave length of $\lambda = 366$ nm. The UV-light focused on the sample is produced by a stabilized high-pressure mercury lamp. A semi-conductor UV-detector fixed on the mobile part of the

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goniometer measures the angular distribution of the UV-light transmitted through the sample. A filter inhibits the transmission of fluorescent light from the sample to the detector. From the detector the signals are transmitted further to an integrating digital voltmeter. The signals of the digital voltmeter are transmitted to a computer. The transmission is correlated with the position of the detector on the goniometer. The computer calculates from the angular distribution of the transmitted UV-light radiation a factor called D. D is a unique (mathematically) uniform and steady function of the ratio nucleic acid contents to nuclear volume (vide infra). The cross section of the light-beam on the tissue samples covers an area of 2 x 0.4 mm^2. This irradiated area provides information averaged out over several hundred thousand cells.

THEORY

In compact tissue the cells are usually packed closely together, and the amount of light scattering at cell boundaries is small when compared with the light scattering at the cell nuclei. Through this phenomenon, the determination of the average volume of the nuclei is possible. It is well known, that the volume of a particle is proportional to forward scattering (7). On the other hand the nucleic acid content of cells is proportional to the extinction coefficient of tissue at a wave length of 366 nm. The average ratio of cell nucleus volume to the nucleic acid content per cell is therefore proportional to the ratio of forward scattering to extinction coefficient. This provides the diagnostic information. The radiation distribution in a layer with uniformly distributed scattering and absorption centers is described by the radiative-transfer-equation (Chandrasekhar). The equation is as follows:

$$\mu \cdot \frac{\partial I(\mu,z)}{\partial z} = -\alpha I(\mu,z) + \int_{-1}^{1} d\mu' \mu' I(\mu',z) \int_{0}^{2\pi} d\varphi' i(\mu,\varphi;\mu',\varphi')$$

with $\mu = \cos \vartheta$. (1)

Here radiation intensity I is given as a function of a coordinate z perpendicular to the layer surfaces and as a function of the inclination angle ϑ in regard to the so called z-axis. The expression $(\mu,\varphi;\mu'\varphi')$ is called the scattering function and is defined in the thin layer. The angle between the directions characterized by ϑ,φ and ϑ',φ' is marked by Θ. $i(\mu,\varphi;\mu',\varphi')$ can be described by a series expansion:

$$i(\mu,\varphi;\mu',\varphi') = \alpha_s \sum_{n=o}^{N} f_n \cdot P_n(\cos\Theta) = \alpha_s \, i_o(\Theta). \quad (2)$$

Additionally, the following norm must be chosen:

$$\int_{-1}^{1} \sin\Theta \, d\Theta \int_{o}^{2\pi} d\Phi \, i_o(\Theta) = 1 \quad (3)$$

The $P_n(\cos\Theta)$ are the legendre polynomials, α_s is the scattering coefficient. The following ansatz solves Equation (1):

$$I(\mu,z) = U(\mu) \cdot V(z). \quad (4)$$

Using Eq. (4) we obtain Eq. (5), which describes radiation emerging from an optically thick layer (y is a characteristic parameter of the layer):

$$I(\mu) = \frac{\text{const}}{(1-y\cdot|\mu|)}. \quad (5)$$

Equation (5) is valid in the case of strong absorption for small angles ϑ and for boundary layers without reflections. By means of the addition theorem and the orthogonality properties valid for legendre polynomials we obtain a relation between y and β, the so-called albedo for single scattering:

$$\beta = \frac{2}{\sum_{n=o}^{N} q_n \int_{-1}^{1} \frac{P_n(M)}{(1+yM)} dM} \quad (6)$$

with $q_n = 4\pi f_n \cdot h_n(1/y)$ (7a)

and $q_o = 1$ (7b)

β is defined as ratio of scattering to extinction coefficient.

The $h_n(1/y)$ are polynomials defined by Mika. Measurements on tissue samples indicate:

$$y \approx 1. \quad (8)$$

In this case the following is valid:

$$h_n(1/y) \approx (-1)^n \quad (9)$$

From (6), (8) and (9) we obtain:

$$\beta \cdot 4\pi \cdot \sum_{n=o}^{N} fn = \frac{2}{\int_{-1}^{1} \frac{1}{(1+yM)} dM} \quad (10)$$

β is defined as ratio of α_s to the extinction coefficient α. By means of Eq. (2) we see, that the left side of Eq. (10) is proportional to the ratio forward scattering ($\Theta = 0$) / extinction

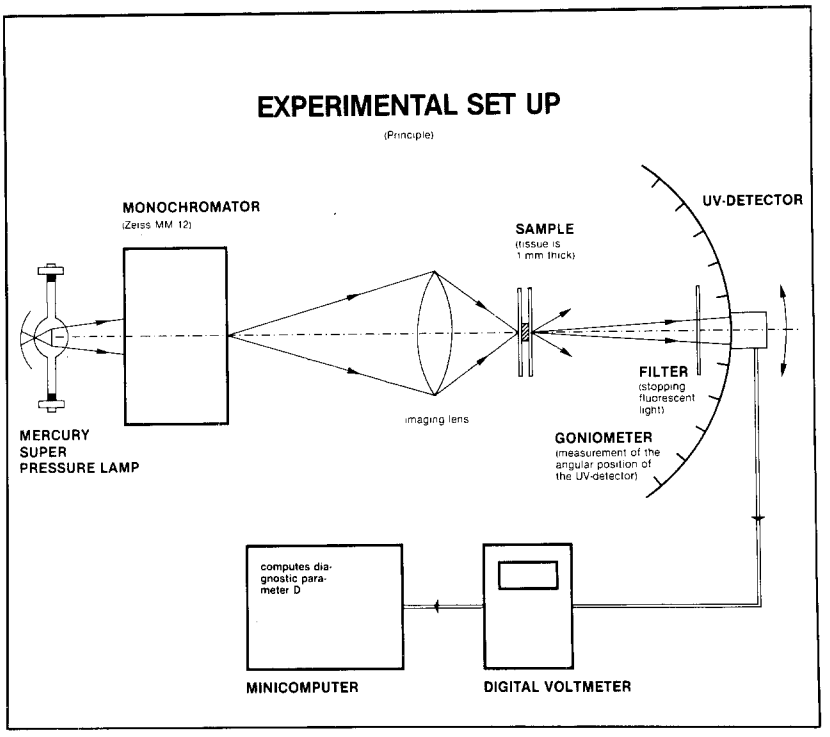

Fig. 1. UV-light is produced by a mercury super pressure lamp. The focused monochromatic UV-beam is perpendicular to a sample mounted in the centre of the goniometer. A UV-detector measures the angular distribution of the light transmitted through the sample. A minicomputer calculates the so-called parameter D containing the diagnostic information

coefficient. Thus, the left side of equ. (10) is proportional to the average ratio of cell nucleus volume/nucleic acid contents as discussed earlier. We see from Eq. (10) that 1-y is a unique, uniform and steady function of the left side of (10). 1-y provides the same diagnostic information as the average ratio of cell nuclear volume/nucleic acid contents per cell. For simplification we defined parameter D:

$$D = 1-y \qquad (11)$$

In our experience the homogeneous layer described above is rarely found in mammalian tissues. More frequent are layers which are weakly heterogenous. In this case y is replaced by $\bar{y}$. We can determine $\bar{y}$ experimentally as follows:

Using Eq. (5) a first y-value is determined from measurements of the intensity for $\vartheta = 0^{o}$ and $\vartheta = 3.3^{o}$; one can call this value y_1. In the same manner can be obtained a second y-value, by means of intensity measurements for $\vartheta = 0^{o}$ and $\vartheta = 6.6^{o}$. This y-value is called y_2. In general y_1 is not equal to y_2. From y_1 and y_2 one gets $\bar{y}$:

$$\bar{y} = \frac{y_1 + y_2}{2} \qquad (12)$$

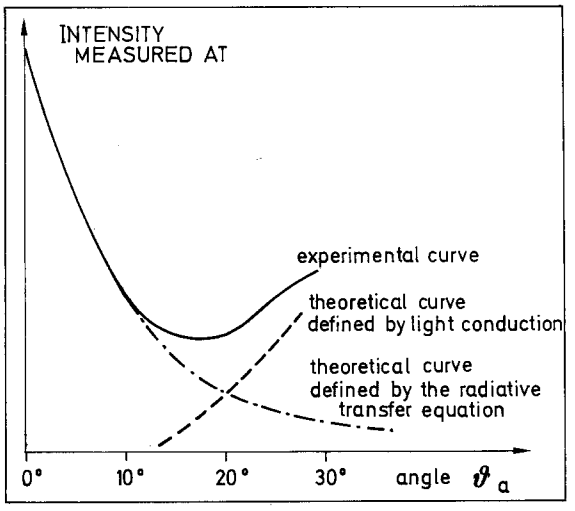

Fig. 2. Radiative transfer through a thick layer (experimental curve) can be explained by superposition of the theoretical curves defined by light conduction and by the so-called radiative transfer equation. In the angular interval between 0^{o} and 12^{o} light conduction is suppressed by squeezing the tissue. From measurements in this interval the diagnostic parameter D is calculated using radiative transfer equation.

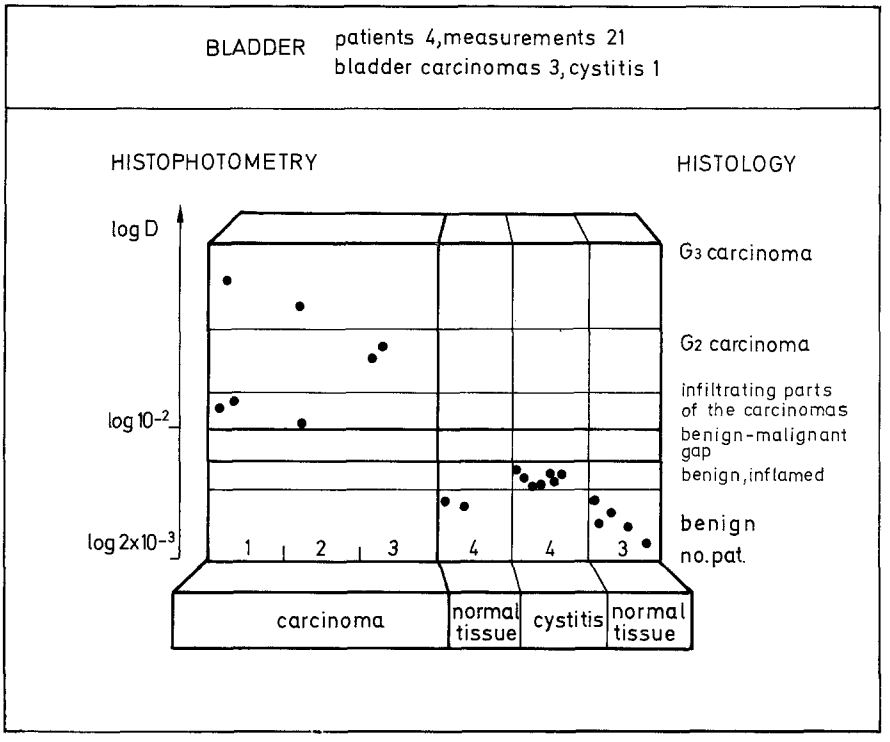

Fig. 3. On the left of the diagram the logarithm of the diagnostic parameter D is specified. D was calculated from the measurements by means of the extended radiative transfer theory. Underneath the patient number is listed and on the right side of the diagram the histological diagnosis. All tissue samples were examined histologically after measurement

The radiation emerging from a weakly heterogenous layer can be described mathematically by superposition of solutions for homogenous layers. The replacement of y by $\bar{y}$ can be explained with this manipulation. The large majority of tissue is filled with channels, e. g. blood and lymphatic vessels and glandular ducts. The interaction between light and channels produces 2 effects:

a. scattering by the channels,
b. light conduction through the channels.

In a few instances the channels are so numerous in the tissue, that light scattering is appreciable. It alters parameter D in a characteristic manner and provides additional diagnostic information on the nature of the tissue. D is then determined according to the formulas for the heterogenous layer.

Light conduction through continuous tissue channels produces a striking effect. A method has been found to sufficiently suppress the light conduction for small angles ($0^{o} \leq \vartheta < 12^{o}$), so that only light produced by interaction with the cell nuclei is transmitted.

Suppression of light conduction is achieved by squeezing the tissue. Squeezing causes, on the one hand, marked deformation of the channel walls (e. g. ripples), and on the other hand, a wider alignment of the channel axis resulting in larger angles.

The theory permits a statement on the degree to which the tissue must be squeezed in order to achieve negligible small angle light conduction. The experiment confirms this theory: When an approximately 1 mm thick sample of prostate tissue rich in glandular ducts is pressed in the way calculated from the theory and the transmitted radiation is then measured, the curve shown in Fig. 2 is obtained (solid line). The marked minimum of the curve at 18^{o} suggests the absence of light conduction in the small angle region.

MATERIALS AND MEASUREMENTS

Tissue samples from 3 patients with bladder tumours were collected by TUR, and from one patient with a bladder diverticulum by transabdominal surgery. The samples from these 4 patients were measured after fixation in formalin.

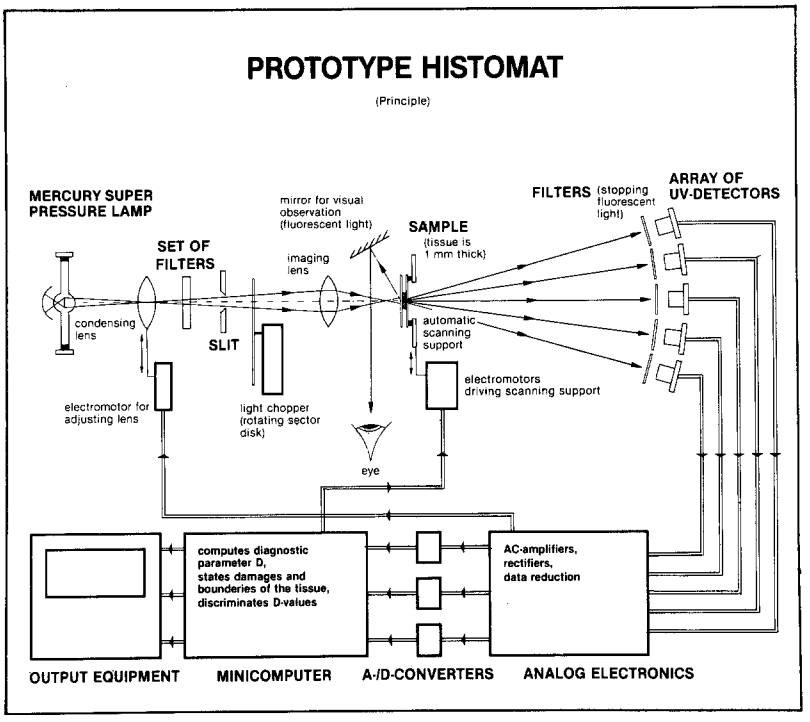

Fig. 4. Scheme of the prototype "Histomat" which drastically reduces the diagnosis time to 25 seconds. The diagnostic parameter "D" is determined by fully-automated measurement and calculation. Goniometer arrangement of the experimental set-up (Fig. 1) is replaced by a detector array.

Twenty one measurements were performed 48 h or later after fixation. To ensure a stable measuring state the high mercury pressure lamp was connected to a stabilised power supply. Before and after measurements the dark effect, i. e. the voltage in the system without radiation input, was determined. Next the reflection and the refraction of the transmitted radiation at the surface of the glass slide were considered and corrected (Fresnel-formulae). The refraction index of tissue and glass can be considered to be equal. Finally the measurements were calculated for a spot-like detector. Then the parameter D (called diagnostic parameter D) was evaluated according to the section theory. Measurements and evaluations take about 180 minutes per sample. The measurements were carried out in a blind study and were compared with the histological diagnosis.

Experimental Results

The results of this simple study for bladder tissue can be seen in Fig. 3. On the left of the diagram the logarithm of D, the diagnostic parameter is indicated; underneath the patient number and on the right the clinical and histological diagnosis can be seen. In the diagram the gap between benign and malignant tissue is striking; this results when the measuring beam focuses on clearly benign or malignant tissue. A second significant feature of figure 3 is the clear relationship between D and the clinical-histological diagnosis indicating their complete conformity.

Comparing the histological diagnoses with our quantitative results a third point of interest was found: the possibility of differentiating between infiltrating and non-infiltrating areas of bladder tumours. It was also possible to discriminate between inflamed and non-inflamed tissue.

DISCUSSION

The technique described is applied to solid tissue samples and is therefore called histophotometry, Numerical results are obtained by this method. By comparing these results with histological findings a clear correlation was found and this has been true also for other tissues from the GU-tract. With the method used, the calculations and measurements were rather time-consuming. This limited the number of samples tested. It is therefore intended in the future to utilise the recently developed prototype (figure 4) which reduces the time of measurements

and evaluation to 25 seconds in contrast to 180 minutes. Measurements can also be carried out, without any preparation of the tissue, e. g. during surgery.

The present investigations have confirmed previous results obtained by Goerttler et al. (3) indicating that the correlation between nuclear volume and the nucleic acid content per cell is a reliable parameter in discriminating between benign and malignant tissue. Additional information has been produced about tumour infiltration and inflammatory reactions.

There is a marked gap between benign and malignant bladder tissue. Histologically patients 1 and 2 showed high grade G_3 bladder-carcinoma of the transitional epithelium with infiltration of the muscle (T_2 to T_3). In patient 3 no signs of muscle infiltration could be established histologically in the large exophytic tumour weighing more than 240 gram. The malignancy has been graded G_2. The measurements show a marked quantitative difference between G_2 and G_3 tumours. Thus, it seems, that with this new technique the degree of malignancy can be determined.

In contrast to cytophotometric studies histophotometry produces no overlap, but clearly distinguishes the tissue being studied. By cytophotometry the presence of cells with increased nuclear DNA above the normal diploid level can be shown. A general correlation between the degree of heteroploidy and degree of clinical malignancy seems to exist. However, the cytophotometric measurements do not deliver uniform results. Heteroploid cells were also found in normal cell populations and in populations which were only suspicions of malignancy (11). In 1968 Wied et al. (10) introduced the Taxonomic- Intra-Cellular Analytic System (TICAS) for cell indentification by computer, based on scanning microphotometry. The computer discrimination between benign and malignant cells from urine samples is possible with a high degree of accuracy by the TICAS routines and subroutines. The error of classification reaches 10% (5).

In conclusion it seems possible with the technique, to differentiate between normal and malignant bladder tissue, infiltrated and non-infiltrated tissue, and between inflamed and non-inflamed tissue. A fully automatic device which is under development will allow rapid examination of tissue samples.

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Addendum: The prototype "Histomat" was now used. Results were obtained which confirm Fig. 3.

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