VISUAL MICRODENSITOMETRY WITH INSTANTANEOUS STATISTICAL CONTROL IN HISTOCHEMISTRY

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The ability of the specialist's visual system to assess optical properties of an image is extremely great and, what is particularly important, it is capable of learning and development. When visual estimation is used in histochemistry, strict and objective control is essential. A method and apparatus for visual evaluation of optical density in histochemical preparations with instantaneous statistical control are suggested below.

EXPERIMENTAL METHOD

Sections with histochemical reactions for isocitrate dehydrogenase (9) and succinate dehydrogenase (4) in the myocardium of the left and right ventricles (LV and RV, respectively) of four control (C) rats and in one rat 7 days after ligation of the descending branch of the left coronary artery in the juxtaischemic zone (JI) and in intact areas (IA) of the left ventricle were subjected to visual estimation. These five experimental points will be described in the text as follows: 9 LV C, 9 RV C, 4 LV C, 9 LV JI, and 9 LV IA. Two slides were examined from each animal and 50 measurements were made on each slide. Altogether, 28 slides were examined and 1400 measurements made.

A special grid (Fig. 1) was introduced into the ocular of the microscope. It consisted of 16 equal squares, the outlines of which formed 25 right-angled intersections. Two additional lines A and B were drawn, and their intersection distinguished the "reference probe" AB. Near each of the remaining 24 intersections the operator distinguishes visually "working probes," approximately equal in area to the "reference probe" (these are shown in Fig. 1 by a broken line, none exist on the grid itself). Optical density, as the analog of intensity of the histochemical reaction, is estimated in the area of each probe by conventional numbers. A scale of 4 or 5 numbers is most convenient, such as: 0) no reaction, 1) low, 2) average, 3) high, and 4) very high enzyme activity.

The image is introduced randomly in the visual field and the probes are arranged relative to it randomly also. Visual estimations of density in the successive probes (as the eye moves across the grid from left to right and from top to bottom) are noted down so that groups of 10 single measurements are formed. For each group of 10 the arithmetic mean M_{10} is determined, and this subsequently constitutes the "counting unit." The groups of 10 are analyzed at the rate of about 10 groups in 3-5 min. For convenience, the numbers are represented in the text by whole numbers (i.e., the recorded values are multiplied by 100).

The necessary and sufficient number of M_{10} in each series of measurements is determined in the course of the work on a statistical basis: The mean must be determined with a reliability of close to 90%. The following means were determined: 1) for a single slide – M_S; 2) for a single animal – M_a; 3) for one experimental point (one or several animals under identical experimental conditions) – M_p.

The difference (similarity) between two compared means was determined by the divergence of the confidence limits for a 95% level of significance. The confidence interval was calculated by Strelkov's method [2]: $L = a\mathrm{Rn}$, where a is the amplitude of the variance series (the difference between its largest and smallest terms), and Rn is Strelkov's coefficient for the given number of terms in the series, i.e., the number of M_{10}. Reliability was calculated by the equation:

$$A = \left(100 - \frac{100L}{M}\right)\%,$$

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TABLE 1. Ratios Between Different Sections Measured Visually and Instrumentally

Values of M_p compared	Visual measurement	Instrumental measurement
9 LV JI: 9 LV C	1.42 times greater	1.38 times greater
9 LV JI: 9 LV IA	1.25 times greater	1.45 times greater
9 LV C: 9 RV C	No difference	No difference
9 LV C: 4 LV C	1.55 times greater	1.58 times greater

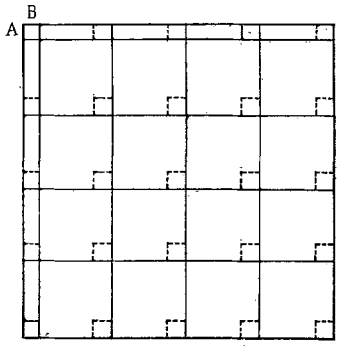

Fig. 1. Scheme of grid.

where L is the confidence interval and M the mean. The necessary and sufficient number of measurements gives A = 90%; if the number of measurements is excessive A > 90%, if it is insufficient A < 90%.

EXPERIMENTAL RESULTS

With one slide with reaction 9 LV C, 5 values for M_{10} were obtained: 260, 250, 240, 260, and 260. This variance series has $A = 260 - 240 = 20$, $R_5 = 0.55$, $L = 20 \times 0.55 = 11$, the slide mean $M_S = 254 \pm 11$, and A = 96%.

The differences between densities in the area of one section depends both on the real distribution of densities and on subjective errors of their estimation. Judging from the reliability of the value of M (96%), these are satisfactorily compensated with the method of measurement used. The number of measurements (5 groups of 10) was formally insufficient for slides* (A = 82-87%), sufficient for 8 slides (A = 89-91%), and excessive for 13 slides (A = 92-97%).

In all pairs there was no significant difference between the slides with a reliability of the order of 94%. Consequently, differences between slides due to variation in the conditions of preparation of the sections were completely compensated by this method of measurement.

Differences between individual animals are easily taken into account in this method: $M_a = (M_{S1} + M_{S2}) : 2$. Within the limits of each reaction, M_a of four control animals did not differ significantly with a reliability of the order of 92%, which proves that the results are satisfactorily reproducible by visual estimation.

Mean values of M_p of the experimental point were obtained by averaging of the control for four animals: 9 LV C – 247 ± 10; 9 RV C – 254 ± 17; 4 LV C – 164 ± 18. The myocardium of a rat with experimental ischemia had $M_p = 352 \pm 10$ in JI and $M_p = 282 \pm 22$ in IA. All five values of M_p showed a reliability of considerably more than 90%.

Values of M_p were obtained for the same slides on the basis of measurements made on the TsIM-2 microphotometer [1]. Since the units of measurement are not comparable, ratios between M_p obtained for visual and instrumental estimations separately were studied (Table 1).

It will be clear from Table 1 that visual estimation and instrumental measurements were completely comparable. In a real experiment, when reliability is calculated in the course of sampling of the data, the work is stopped when the planned levels of reliability are achieved. In that case, the calculations were consciously deferred until the end of all the measurements in order to avoid any bias in the results. Reliability for some sections was thus slightly underestimated, and for others it was overestimated.

*The number was omitted in the Russian original – Consultants Bureau.

Consequently, the method of visual evaluation with instantaneous statistical control can be used in histochemical reactions when the investigator can construct a subjective scale of gradations of the feature concerned. In reactions in which proportionality does not exist between optical density and the quantity of indicator, this method is suitable only for use in comparing standard preparations.

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AN ACCURATE AND RAPID METHOD OF ASSESSING ACTIVITY OF CHOLERETICS IN RATS

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There is no simple method for the rapid and accurate detection and screening of choleretics, for practical purposes, under experimental conditions. Choleretic activity of drugs under acute experimental conditions is usually studied in rats by Heidenhain's method [5]* or in one of its modifications [1-4]. These methods consist essentially of the introduction of a cannula into the main bile duct of anesthetized rats, through which the bile is collected. A disadvantage of these methods of testing choleretics is the presence of a foreign body in the bile duct, namely the cannula, which injures the tissue or causes constant irritation of the biliary tract, and this is often reflected in the experimental results.

The method now suggested eliminates these defects and, together with its simplicity in use and its accessibility, it always gives stable results. It is essentially as follows. The experimental rat is anesthetized with ether and urethane, fixed on an operating table, and laparotomy is performed through an epigastric incision 1.5-2 cm long. The duodenum and the point where the bile duct enters it are then located. Above this point the duodenum is ligated. Another ligature is tied below the point of entry of the bile duct into the duodenum, into the lumen of which one end of a rubber cannula-tube 1 mm in diameter and 4-4.5 m long is first introduced. A closed sac with a volume of 0.5 cm^3 is thus formed from the duodenum. Bile escapes from the bile duct into this sac and then into the cannula-tube (Fig. 1). The cannula-tube (a transparent rubber capillary tube) is first calibrated by means of a tuberculin or insulin syringe, and then wound spirally on a stand on which a scale is mounted, with divisions corresponding to calibration of the capillary tube in millimeters. In the case illustrated, the value of each scale division was 0.025 ml of the volume of the capillary tube (Fig. 1). As a result of pressure in the bile duct and in the isolated duodenal pouch, and also of the properties of the capillary tube, bile constantly fills the special reservoir (capillary tube) as it enters the duodenum. Bile collecting in the tube is measured hourly according to the scale readings for the necessary period of time. At the end of the experiment the cannula tube, filled with secretion, is cut from the duodenum, and its contents, either as a whole or in hourly portions, are subjected to biochemical analysis.

To prevent the evacuatory function of the gastrointestinal tract, an anastomosis is formed by means of a rubber tube between the proximal and distal parts of the duodenum (Fig. 1).

The duration of the operation to form the isolated sac, to introduce and fix the cannula tube, and to form the anastomosis was 5-7 min per rat. In the course of one working day the choleretic activity of one preparation can be studied in 14 rats (including seven controls) by the suggested method and the qualitative and quantitative characteristics of the preparation can be obtained.

*There is no reference 5 in the Russian original – Consultants Bureau.

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