High acutance with improved contrast in black-and-white photomicrography at low magnifications

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The purpose of this communication is to describe the methods used in this laboratory to improve the resolving power, apparent sharpness and contrast in black-and-white photomicrographs of sections stained with hematoxylin and eosin (H&E) at low magnifications (×25 to ×40). The combination of a soft-working, high-definition film developer and a high-contrast copy film has produced superior photographs at all magnifications used.

Film

The emulsion used was Kodak high contrast copy film 5069, available in 35 mm format in rolls of 36 exposures or in bulk rolls of 15.4-meter lengths.

Microscope

Photomicrographs were taken with a Reichert Zetopan research microscope. The manufacturers instructions for setting up the microscope were followed. A Kodak Wratten filter no. 11 (yellowish-green) was used to provide good tonal reproduction and a polarizing filter was used to minimize light scatter.

Exposure

Test strips of high contrast copy film were exposed to H&E-stained tissue sections by trial and error to establish an optimum meter setting for the film developer used. The test negatives were assessed by making prints. The negative exposed to a meter setting of 25, using an automatic (average exposure) metering device, produced acceptable prints at ×100, ×400 and ×1000.

Average light-meter exposures were made of tissue sections with an automatic exposure system at low magnifications (×25 and ×40). Figure 1 illustrates a ×25 photomicrograph which was exposed in this manner. It shows a granulomatous lesion in a rabbit lung. A Kodak Wratten filter no. 11 and a polarizing filter were used. It was not possible to obtain the degree of overall contrast required in the print. The subtle tonal differences of the cellular elements and the desired rich, brilliant blacks are missing. The best possible negative was not obtained with the use of an average exposure method at this magnification.

To correct this problem, the zone system of exposure using a place-fall technique was applied (Adams, 1968). The zone system for black-and-white film emulsions is made up of ten discernable steps in a gray scale from zone 0 (complete lack of density) to zone IX (white without texture). Light meters are designed to integrate the various light intensities striking the sensor to produce a product value of middle gray. Middle gray is represented as zone V.

In order to obtain the desired photographic quality of contrast and increased tone differentiation in low-power photomicrographs, the single tone value of the background (light source) was metered. The automatic exposure system was replaced with a camera back having a built-in, manually controlled light meter. A Wratten filter no. 11 and a polarizing filter were kept in the microscope light path for all exposures. A clean glass slide was placed on the microscope stage

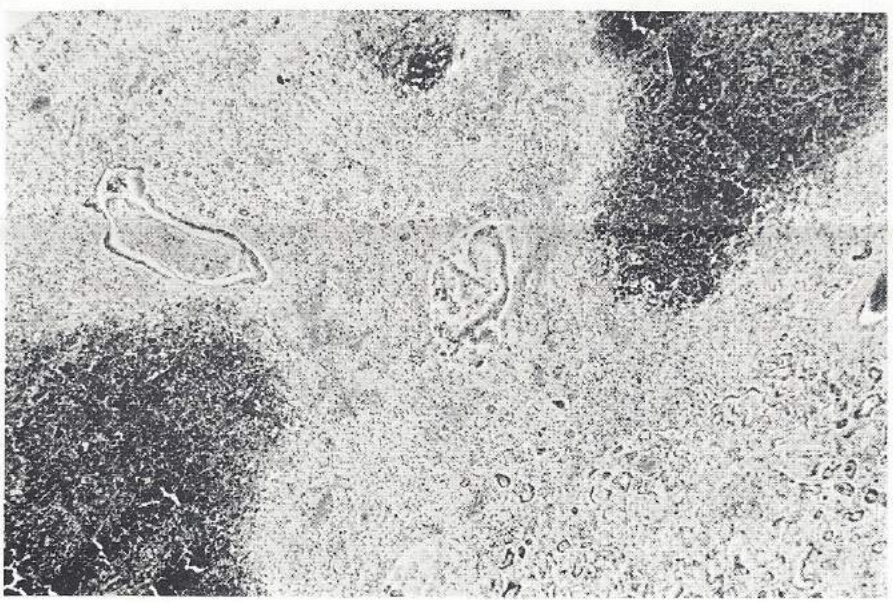

Figure 1—A X25 photomicrograph of an H&E-stained section of a granulomatuous lesion in a rabbit lung. An average (zone V) exposure was given through a polarizing filter and a Kodak Wratten filter no. 11 on high contrast copy film. Subtle tonal differences of the tissue elements and rich brilliant blacks are missing. General contrast is poor.

to eliminate the light loss through the glass as an exposure variant when an actual tissue section slide was placed on the stage. A scanning condensor was used on the microscope for low-power microscopy, and this provided a wide area of even light distribution.

The absence of diaphragms on this condensor eliminated them as possible exposure variants. Line voltage fluctuations resulting in shifts of the microscope lamp intensity were eliminated with the use of a voltage stabilizing transformer. Film exposure was controlled only by light intensity and by the shutter speed on the camera.

Camera movement caused a problem at shutter speeds of 1/8 second and slower; the movement was caused by extraneous vibrations in the laboratory. It was eliminated by increasing the light intensity in the microscope and decreasing the exposure time to obtain an exposure equivalent to that of an average exposure at a meter setting of 25. It was found that a light intensity increase for a meter setting beyond a factor of 4 would considerably shorten the life of the bulb.

To adjust the exposure meter indicator for an average exposure of the background, the meter setting was shifted upwards by the factor of 4 from 25 to 100. The shutter speed for an average exposure of the background ranged from 1/250 to 1/125 second for ×25 and ×40 respectively. The shutter speed dial was the only exposure control used for the subsequent place-fall method test exposures when the light intensity was set for the desired magnification.

The microscope and camera was set up at ×40 for the place-fall test exposures. A shutter speed of 1/125 second represented a zone V exposure of the background. The clean glass slide was replaced by an H&E-stained tissue section slide. The slide was focused and composed. A series of exposures was made that placed the background gray value for each frame into a progressively lighter zone. The series consisted of frame 1—1/125 for zone V; frame 2—1/60 for zone VI; frame 3—1/30 for zone VII; frame 4—1/15 for zone VIII; and frame 5—1/8 for zone IX. Four rolls of film were exposed in this manner.

The recommended developing time at 20°C for the film developer used varied from 7 to 10 minutes. The first exposed film was developed for 7 minutes, the second for 8 minutes, the third for 9 minutes and the fourth for 10 minutes. The developer temperature for all films was maintained at 20°C. The films were fixed, washed and dried in a similar manner.

Density measurements of the negatives were not possible because a densitometer was unavailable. Assessments were made from the best possible print of each negative. The print from frame No. 3, zone VII on the film developed for 10 minutes was selected as being optimum.

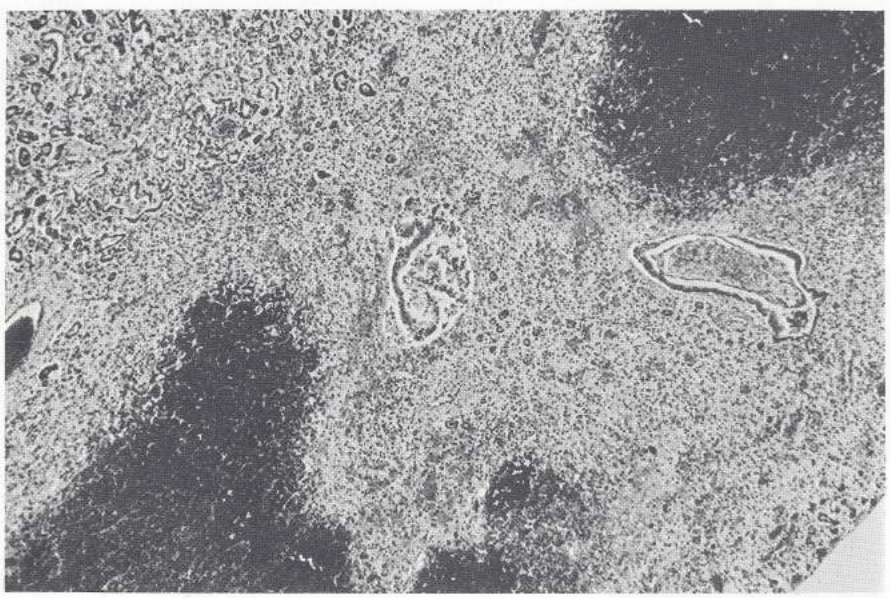

Figure 2—The same area of the section as Figure 1. The film was Kodak high contrast copy film. The exposure was made to place the background light in zone VII. The gray values of the section are no longer subject to a compromise exposure between the high and low densities of the cellular elements.

Figure 2 is an illustration demonstrating the place-fall technique used at ×25. An exposure of a 1/60th second placed the background in zone VII (very light gray). The negative was developed for 10 minutes. The tone values of the tissue fell into a precise relationship to the very light gray background. Much greater cellular detail was evident. Rich blacks and a good overall tonal range were easily obtained when the negative was printed on a Polycontrast rapid RC paper with a printing contrast filter value of 3½.

Other negatives were exposed at ×25 and ×40 for a variety of tissue sections of various organs. In all exposures, the background was placed in zone VII. Subsequent printing of the negatives confirmed zone VII background as the optimum highlight value for the shortened tonal range of high contrast copy film.

Film developer

A high-definition (high-acutance) developer was used for all film development to maximize the definition in the negative image. The developer is described as formula No. 105 (Jacobson & Jacobson, 1972, p. 215).

The developer consists of these chemicals:

Metol	–	5 gms
Sodium sulphite, anhyd.	–	25 gms
Sodium carbonate, anhyd.	–	25 gms
Water to make	–	1000 ml

The developer is mixed as a stock solution. Dilution for use is one part developer to ten parts water. The development time (Jacobson & Jacobson, 1972) ranged from 7 to 10 minutes to 20°C. The developer is characterized by the absence of bromide, which enhances local developer exhaustion at the boundaries between high and low density areas. Since the diluted developer has a low concentration of developing agent, which is quickly exhausted after one use, reuse of the working solution resulted in severe underdevelopment of the high contrast copy film.

Development agitation was by inversion. After several inversions at the beginning of the cycle, the tank was tapped against the edge of the sink to remove air bells clinging to the film emulsion. Thereafter, for the remaining time of development, the tank was inverted once per minute. Agitation of this developer was carefully controlled because too vigorous agitation neutralizes the adjacency effect.

Paper

Kodak Polycontrast rapid RC paper, medium weight, surface F, was chosen as the enlarging paper because of its variable contrast capabilities, the whiteness of it's base, the shortened fixing and washing time and its capacity for drying without heat to a high gloss, due to its resin coating. A Kodak Polycontrast filter kit (Model A) was used to control print contrast.

Paper developer

Kodak Dektol developer, diluted 1:2 is the recommended developer for Kodak Polycontrast rapid RC paper. The suggested developing time is from 1½ to 3 minutes. When additional contrast control in printing was deemed necessary, it was provided by Beer's two-solution variable contrast paper developer (Adams, 1968). Dilution ratios of stock solutions A & B of Beer's developer mixed with water provided a variable range of working solutions from low to high contrast. The developing agent in solution A is metol and the developing agent in solution B is hydroquinone. The useful development time was found to be 1 to 3 minutes. Prints were fixed, washed and dried in the usual manner.

Print after treatment

When an undesirable degree of graying in the background of prints was evident, a print reducer was used to clear these areas (Adams, 1968). The reducer was designed to clear the whites of fog. The reducer was mixed and stored in two parts:

Part A: Water .....300 cc.
Potassium ferricyanide ..62.5 gms.
Potassium metabisulphite
or sodium bisulphite .....4.2 gms.
Water to make .....500 ml.
Part B: Water .....600 cc.
Ammonium thiocyanate ..330 gms.

Potassium bromide .....30 gms.
Water to make .....1000 ml.

One part of solution A and two parts of solution B are mixed with 10 to 15 parts of water immediately before use, since the working solution does not keep. Prints to be cleared in the reducer should be thoroughly dried before the bleaching process is started. Using a wet print can cause the bleach to attack the lower and mid-tones. A solution that is too dilute can also cause reduction in the lower and mid-tones.

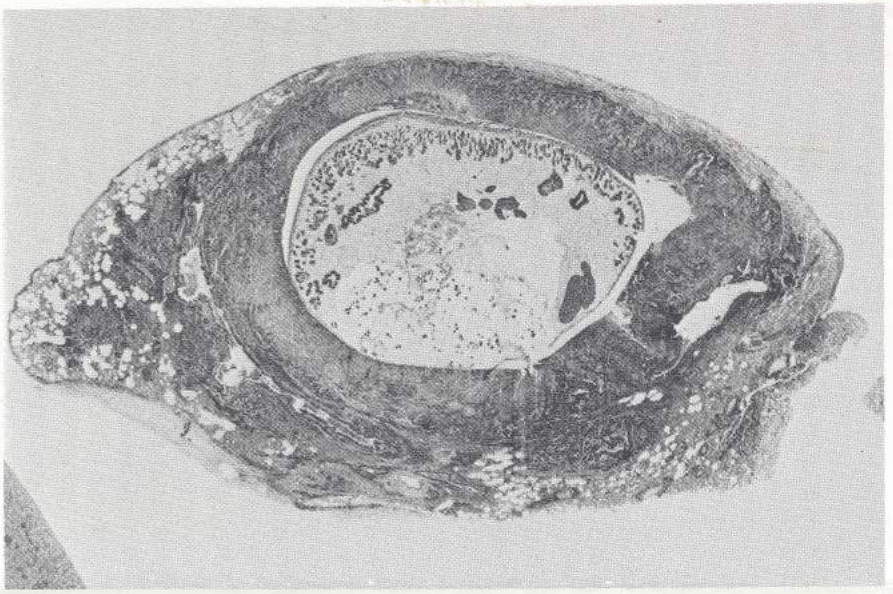

Figure 3—Whole tissue section of a lung fluke in the lung of a striped skunk; X7.7. Photographed on Ilford FP4 film with a 50 mm macrolens and transmitted light on a light table. The photograph lacks contrast, and fine detail is not sharp.

A dry print was quickly slid edge first and face-up into the bleaching solution. The print was agitated vigorously. A great deal of care was always necessary to prevent the reduction of highlight detail. When the bleaching was completed, the print was quickly removed and immediately rinsed under running tap water to remove excess solution. It was then washed in running water for a few minutes. No further treatment was necessary. The print was then dried in the usual manner.

In this laboratory, the working solution is always made up with 15 parts of water. The length of time required to obtain the desired degree of clearing ranged from less than 5 seconds to 15 seconds. No evidence of uneven reduction was noted.

Whole-section photography

The reproduction of whole sections of tissue was greatly improved by the utilization of a Repronar, (Honeywell, model 805A). The film and developer used for photomicrography negative production was used. Trial and error exposures indicated Repronar settings of 18 for the film value and the electronic flash set to "low" position. A Kodak Wratten filter no. 11 (yellowish-green) was used. The aperture setting was $f/7.8$ for a $\times 1$ exposure. Bracketing exposures were done for excessively thin, lightly-stained tissues, or for thick, heavily-stained sections. When whole-section photographs were taken, the best results were obtained when tissue sections were mounted between 51 millimeter-square glass slides. Tissues mounted on standard microscope glass slides were adequate to use with the Repronar, but opaquing of the negative to remove unwanted areas was necessary on some of these.

To demonstrate the improved reproduction of whole sections, a photograph taken using an earlier method (Figure 3) is compared with one taken using the method described here (Figure 4). Figure 3 is a whole-tissue section of a lung fluke (*Paragonimus kellicotti*). The section was photographed with a 50 mm macrolens and transmitted light on a light table. Ilford FP4 film was used. The meter setting was 200. Ilford Microphen developer was used. The negative was printed on Kodak Polycontrast rapid RC paper through a no. 4 printing filter. The paper developer was Beer's solution no. 5 (moderately high contrast). The overall contrast is poor. Fine detail in the structure of the parasite and bronchial wall is not sharp. Tonal separation is lacking.

Figure 4 illustrates the same section when photographed with the Repronar. Kodak high contrast copy film 5069 was used and processed in film developer no. 105 at a working dilution of 1:10 for ten minutes at 20°C. The negative was printed on Kodak Polycontrast rapid RC paper through a no. 3 printing filter. The

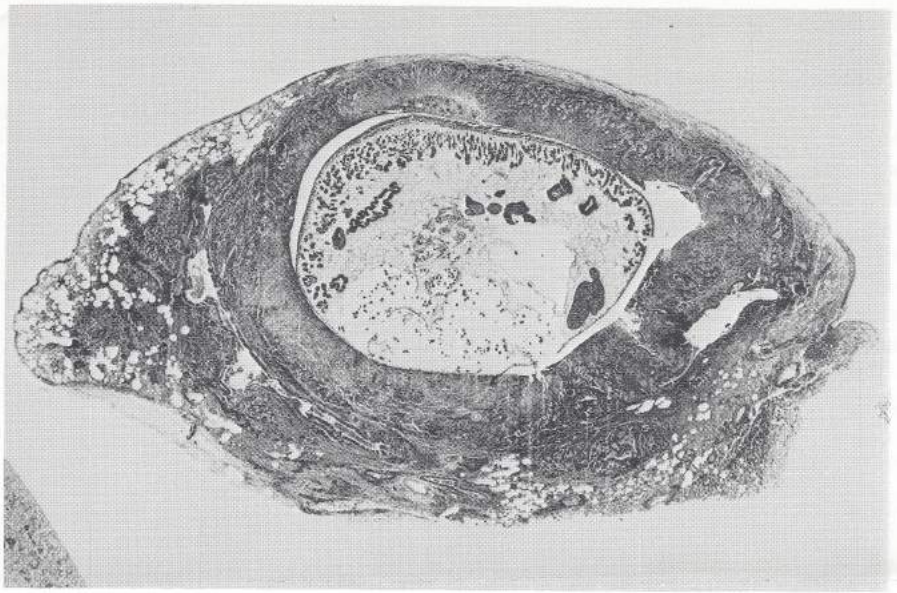

Figure 4—Same section as Figure 3 but photographed on Kodak high contrast copy film with a Repronar and electronic flash. The negative was processed in a soft-working, high-definition developer. Note the greater resolution and improved contrast. The photograph was bleached to clear the greying background.

print was bleached as earlier described to clear the greying background. Note the greater resolution and improved contrast that was obtained even at this linear magnification of ×7.7.

Conclusion

Photomicrographs and whole-section photographs produced using the materials and methods described in this article have consistently yielded a degree of resolution and brilliance not previously attained by us in low-power work.

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About the author—Edward Eaton is a technician doing photographic work in the Department of Pathology, division of Parasitology, and involved in field and clinical work associated with research programs.

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