The photographic development of coloured grains in radioautographs

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Summary

Tissue sections are prepared and coated with NTB 3 Eastman Kodak emulsion for radioautography in the usual way. After they have been exposed for a suitable time they are processed with no agitation in the following solutions, which must be freshly prepared. Place for 3 min at 24° C in a developer solution which contains per litre 40 g sodium carbonate, 2 g sodium sulphite, 1 g potassium bromide, 2·5 g 'genochrome', 40 ml ethylene glycol, and 100 ml acetone in which 2 g of α-naphthol is dissolved. Wash for 2 min in running tap-water. Place for 2 min in a solution containing 30 g potassium ferricyanide and 15 g potassium bromide per litre. Wash for 2 min in running tap-water. Place for 2 min in a solution containing 40 g sodium sulphite, 240 g sodium thiosulphate, and 100 ml commercial formalin per litre. Wash in running tap-water for 2 min. Place in 0·02% neutral red for 5 min. Wash in distilled water for 1 min. Dip in a solution containing 10 g gelatin and 1 g chrome-alum per litre, which has been heated in a water-bath until liquid, and then allowed to cool to room temperature. Stand each slide vertically to dry in air, then mount a coverslip with Canada balsam. This method gives blue grains: if red ones are required substitute *p*-nitrophenylacetonitrile (Eastman Kodak) for α-naphthol.

Introduction

In an attempt to demonstrate the incorporation of tritium-labelled tyrosine into melanocytes, radioautographs were prepared by the coating technique of Kopriwa and Leblond (1962). On examination of the radioautographs it is difficult to see those black grains in the photographic emulsion that are situated over a dark background of melanin. The difficulty is aggravated by the close resemblance, in colour and size, of silver grains to melanin granules. Because the size of both these particles is near the limit of resolution of the light microscope, considerable effort is required to distinguish black silver grains of about 0·5 μ diameter on a background of black melanin granules of about 1 μ diameter. The distance which separates the layer of silver grains from the melanin granules is not sufficient to enable them to be readily distinguished by focusing with a microscope.

A radioautograph in which the grains developed in the emulsion can be readily distinguished from the granules of melanin in the tissue section has been prepared by the photographic development of bright-coloured grains instead of black silver grains.

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Material and methods

The tissue used was skin bearing black hair taken from the neck of hooded rats. When they were 25 days old the animals were anaesthetized with ether and the hair clipped short in the region between the fore-limb and the ear. At this age the skin is pink. When they were 31 days old the skin colour at the ventral edge of the clipped region changed from pink to black. During the following day this colour change spread across the region in a dorsal direction. It corresponds to the second wave of hair growth. The darkening of the skin is produced by new black hair below the skin surface where it is forming in the follicles. The melanocytes in the hair bulbs produce melanin which passes into the hair shafts as they grow. Kukita (1957) showed that the melanocytes are very active while the hair is growing.

When all the skin of the neck was black each rat was given 10μC/g body weight of DL-tyrosine labelled with tritium. The tyrosine was generally labelled and had a specific activity of 434 mC/mM. The rats were killed 30 min after the injection. Skin from the neck was fixed in Bouin's fluid and sections prepared at a thickness of 5μ. When mounted on microscope slides the unstained sections were coated with Kodak NTB 3 emulsion. Similar preparations were made with skin from rats which had not been injected with radioactive tyrosine.

The method was perfected in 3 stages.

Stage I. An old sample of Kodak NTB 3 emulsion, unsuitable for ordinary experimental work, was fogged by exposure to light. It was placed on a darkroom bench in its glass container and exposed to the white room light for 2 sec. After exposure it was thoroughly stirred to distribute the exposed halide evenly through the emulsion. In this way an extremely low level of fog is produced. The fogged emulsion can be stored and used repeatedly.

Clean microscope slides were dipped in the fogged emulsion and dried in the usual manner. They were ready for use as soon as they were dry. It is possible to make such slides in large numbers at short notice. This simple preparation was used until it was possible to make specimens mounted in Canada balsam with coloured grains in the emulsion.

Stage II. Microscope slides bearing non-radioactive tissue sections were dipped into a fogged Kodak NTB 3 emulsion similar to that used in stage I. They were ready for use as soon as they were dry. Again it is possible to make such slides in large numbers at short notice. They were used until it was possible to make preparations mounted in Canada balsam with coloured grains in the emulsion over the tissue sections.

Stage III. Microscope slides bearing radioactive tissue sections were dipped into fresh Kodak NTB 3 emulsion which had not been fogged. These preparations were not ready for use immediately, because they had to be stored for exposure of the emulsion. Because of this delay they are less convenient to use for trials than those of stages I and II.

The solutions used to develop coloured grains are:

Developer

Sodium carbonate	40 g
Sodium sulphite	2 g
Potassium bromide	1 g
Genochrome (May and Baker Ltd., Dagenham, Essex, U.K.)	2·5 g
Distilled water	to 1 l.

This solution is prepared as the first step of the technique. The genochrome must be added after the salts are dissolved.

Colour coupler

α-Naphthol (for blue grains) or *p*-nitrophenylacetonitrile (for red grains)	20 g
Acetone	to 1 l.

Ethylene glycol

Bleach

Potassium ferricyanide	30 g
Potassium bromide	15 g
Distilled water	to 1 l.

Fixer

Sodium sulphite	40 g
Sodium thiosulphate	240 g
Commercial formalin	100 ml
Distilled water	to 1 l.

Counter stain

Dilute 20 ml of 1% neutral red with 1 l. of distilled water.

Gelatin supercoat

10 g best pig-gelatin are dissolved in 500 ml distilled water, and 1 g chrome-alum is dissolved in another 500 ml distilled water. Mix the 2 solutions.

It is best to prepare all the solutions immediately before use. The directions have all been given to prepare 1 l. Suitable adjustment of the volume to be prepared must be made according to the number of slides to be processed.

When everything else is ready for the slides to be processed, prepare the developer. To each 100 ml of developer add 4 ml ethylene glycol and 10 ml colour coupler.

The slides are processed in total darkness until the end of the fixation stage. No agitation is used throughout.

Develop at 24° C	3 min
Wash in running tap-water	2 min

Bleach	2 min
Wash in running tap-water	2 min
Fix	2 min
Switch on light	
Wash in running tap-water	2 min
Counter stain	5 min
Distilled water	1 min
Gelatin supercoat (heat until liquid in a waterbath, then allow to cool to room temperature)	1 min
Wipe the back of the slide until it is clean	
Stand slide vertically until dry	
Mount a coverslip directly with Canada balsam.	

Discussion

For nearly half a century it has been possible to develop ordinary photographs in one colour other than black by a process involving colour couplers (Fischer and Siegrist 1914). The technique for radioautographs described in the present paper does not involve any new principle of photographic development. Its success rests on the choice of a developer which will produce relatively insoluble coloured grains. Most colour developers produce coloured grains (discrete dye spots) which are so readily soluble that they cannot be retained in an emulsion when they are formed under the conditions used in radioautography.

The principle of the photographic development of coloured grains is slightly different from that of the production of silver grains. The developer oxidizes at the site of the latent image in the emulsion when it reduces silver halide to silver. One of the products of oxidation combines with a colour former to produce a dye. When the emulsion is taken out of the developer solution each silver grain is situated within a spot of dye. The silver grain is rendered soluble by a bleach solution. The fixing bath removes the undeveloped silver halide and the bleached silver grains, leaving translucent spots of dye (referred to as coloured grains). It is apparent that the substance retained in the emulsion is derived from the developer and not, as in ordinary development, from the silver halide.

The technique is labile. If the functions of the various components of the developer mixture are appreciated it is possible to control the density and the size of the coloured grains. The conditions of development can be adjusted so that radioautographs are produced which suit the problem under investigation.

All colour developers are unstable and genochrome is no exception. As soon as the developer mixture is made up it begins to change colour because it is oxidized by oxygen dissolved in the solution and taken up from the air. It is for this reason that the developer solution is prepared as the first stage of the technique, when everything else is ready. If this spontaneous oxidation is allowed to proceed for too long before the solution is used, the

concentration of the coloured compound exceeds its solubility and artifacts are produced on the surface of the emulsion.

Another consequence of the developer's instability is the sensitivity of its action to changes of temperature. A small increase in temperature, of about 1° C, results in an appreciable increase in the amount of colour produced in each grain. The highest temperature at which radioautographs can be developed is 24° C. It is determined by the rate at which the gelatine base of the emulsion dissolves. Rapid changes in temperature during processing increase the possibility of the emulsion moving or peeling off the section. Therefore all the processing solutions should be at the same temperature.

Although it would be desirable to reduce the concentration of sodium carbonate in the developer solution because it causes the gelatin of the emulsion to swell, this is not possible because genochrome only works well in a very alkaline solution of pH 10 or above.

It is not advisable to alter the concentrations of sodium sulphite or potassium bromide to modify the action of the developer solution. Sodium sulphite slows atmospheric oxidation of the developer but also competes with the colour coupler for the oxidized form of the developer. So although the presence of sodium sulphite is necessary to retard atmospheric oxidation, its concentration must be restricted to allow the colour coupler to act unhindered. Potassium bromide slows down the rate of development and reduces background fog.

A colour coupler is chosen according to the colour of the grains required. Blue grains are produced by α-naphthol and red grains by *p*-nitrophenylacetonitrile. Blue grains have retained their colour for 2 years but red ones turn black after 3 months. The amount of colour in each grain can be increased by an increase in the concentration of the colour former. The change is produced because the competition between colour former and sodium sulphite for the oxidized developer moves in favour of the colour former when its concentration is increased. Another way to increase the amount of colour in each grain is to increase the time of development.

The ethylene glycol in the developer mixture enables the dye formed to move away from the surface of the developing silver grain. Development is hindered if the dye accumulates there. An increase in the concentration of ethylene glycol results in an increase in the diameter of the dye grains.

The blue dye formed by this technique is soluble in ethanol, as well as in normal and tertiary butanol. Consequently, dehydration of the slides by alcohols in preparation for mounting a coverslip in the usual manner is impossible. When they were mounted from water with alkaline chrome glycerine jelly the blue grains disappeared overnight. The dye is known to be soluble in acid aqueous solutions so it must be sufficiently soluble in alkali for the small amount present in a radioautograph to dissolve. This part of the technique would be simplified by the introduction of a colour developer or coupler, capable of acting on radioautographs which would give dyes insoluble in these solvents.

At present the preparations have to be dried in air before a coverslip is mounted. During this process the photographic emulsion may lift off the section. If this happens an air pocket, which is difficult to remove, is created between the section and the emulsion. To reduce the incidence of this artifact the preparation is given a gelatin supercoat before drying. This extra coat of gelatin covers any holes in the emulsion through which air might enter, and furthermore increases the thickness of gelatin over the slide so that drying occurs evenly.

The method of colour development here described does not produce grains comparable with those produced by the conventional development process. The grains are in fact spots of dye, less uniform in diameter than silver grains. It is not possible to decide whether the larger spots correspond to a single silver grain or a group of grains. It must be stressed that the quantitative nature of this method has not been established. As soon as this has been done it will be possible to refine the technique further.

In addition to its use when working with naturally pigmented tissues, the colour modification of radioautography could be used for tissue sections stained black with materials such as osmium. Also it should be possible to separate the images formed in double exposure methods. One layer of emulsion is exposed to a tissue section carrying 2 isotopes and then developed in one colour to show the activity of both isotopes. The preparation is kept until the radioactivity of the isotope with the shorter half life has virtually disappeared, or coated with colloidin to filter off the emission with the shorter range. The slide is then coated with another layer of emulsion. When this has been exposed and developed for silver grains, or grains of a second colour, it reveals the activity of only one isotope. The radioautographs made from the 2 exposures over the same sections could be distinguished with ease.

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