scopic method proposed by Shinowara and Walters (1963). Oxyhaemoglobin, which sometimes accompanies methaemalbumin in plasma, *e.g.*, after recent haemolysis, is converted by dithionite to haemoglobin, with change in absorption spectrum but oxyhaemoglobin does not interfere in this method because, at the chosen wavelength, oxyhaemoglobin and haemoglobin have identical absorption coefficients. However, the change in absorbance of both oxyhaemoglobin and haemoglobin with change in wavelength at this point is considerable and a check should be made, using a solution of oxyhaemoglobin, that the wavelength setting on the spectrophotometer is correct in this respect.

The validity of the method proposed here depends on the absence of any other substance in plasma which is affected by dithionite with significant change in absorbance at 569 mμ. We have examined in this respect a number of normal and pathological plasmas, including some from jaundiced patients, and have found no evidence for such interference.

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Rapid method for the preparation of replicate microslide tissue cultures to facilitate immunofluorescent identification of unknown virus isolates

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Undoubtedly the slowest part of the identification of virus isolates obtained from clinical specimens is their precise identification by the conventional neutralization test in tissue cultures. The problem lies in the wide range of possible antisera which, particularly in the case of entero-viruses, have to be tested before the homologous, infectivity neutralizing serum is found. Hambling, Davis, and Macrae (1963) and Lim and Benyesh-Melnick (1960) have both reported schemes for the production of enterovirus serum pools to facilitate identification of enterovirus isolates. Both systems have the disadvantage of requiring tedious neutralization tests in tissue culture both at the serum 'pool' stage, and subsequently to distinguish the specific reacting serum within the pool.

The immunofluorescent technique offers a potential speed-up in identification, wherein the same enterovirus pools of rabbit antisera may be used in an indirect, two-stage test with fluorescein-isothiocyanate conjugated anti-rabbit serum in the second layer of the fluorescent 'sandwich'. This technique is well known as a method of tracing viruses growing in tissue culture monolayers, invariably on cover slips placed in Leighton tubes. The Leighton tube cover slip method has several important disadvantages in practice for the identification of unknown agents.

First the cover slips are extremely fragile, and are consequently difficult to manipulate in numbers. Secondly, the problem of identification of an unknown agent by immunofluorescent methods can best be tackled by constant reference to control monolayers, infected but treated with normal rabbit serum in the middle layer of the fluorescent 'sandwich'. Frequent comparison is also necessary with the results obtained with other antiserum pools, in the middle layer, for the criteria of positivity are best established by a process of comparison for any unknown agent.

The cover slip method necessitates frequent changes of microslide for cross reference. These are time consuming and comparison from one slide to another may give misleading results because of batch variations from tube to tube.

The method to be briefly described here makes use of tissue culture monolayers grown in glass rings on microslides. These are easy to manipulate, simplify immunofluorescent staining, and greatly facilitate comparison with control spots for comparative purposes. The method

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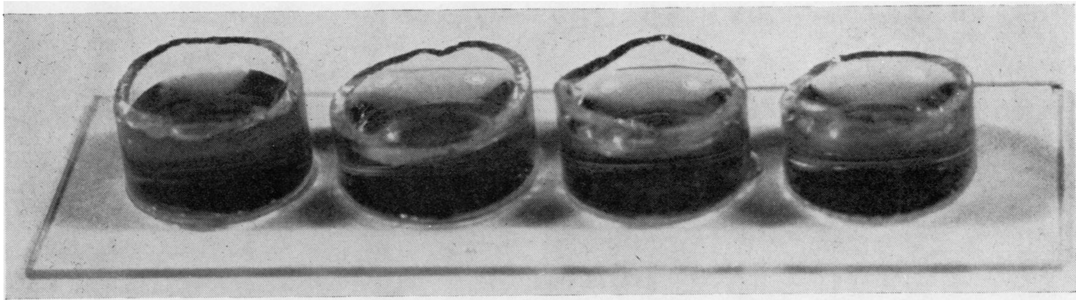

FIG. 1. *Completed microslide assembly with four glass rings in position, each containing tissue culture medium ready for use.*

has been used routinely in this laboratory for the study of virus growth and maturation, and for the identification of fresh tissue culture isolates, for the past two years.

MATERIALS

GLASS RINGS The basis of the technique consists of glass rings cut from tubing of 14 mm. O.D. The tubing has walls 1 mm. thick. The rings are cut 5–7 mm. high and are large enough to allow accurate machining of one end of the cut surface. This end is sanded flat by holding briefly against a rotating fine sand paper disk. The rings are washed in bulk and stored in ethanol at room temperature in screw-capped containers.

MICROSLIDES Any good quality 3 in. × 1 in. microslide can be used. In the author's laboratory special 'fluorescence-free'[1] glass slides are used routinely, because it was found that these have negligible thickness variation. Being 'fluorescence-free', any minute scratches do not fluoresce in blue light when examined in a fluorescence microscope. The slides are washed and stored in ethanol.

METHOD The slides are removed from alcohol and flamed to sterilize. They are assembled inside sterile containers (usually 9 in. phage-typing dishes). Each ring is picked from the jar of alcohol and flamed. It is held in such a way, in forceps, that the ground side can be brought in contact with the microslide. While hot from flaming the ground side is touched gently on the surface of a mixture of equal parts paraffin wax and petroleum jelly. The heat from flaming melts only enough of the wax mixture to coat the ground end of the ring, and the wax remains molten long enough to allow the ring to be pressed gently, without sliding, onto the microslide. The wax solidifies instantly and the ring is held in place with a waterproof joint. Each microslide has a total of up to six rings fixed as described along its length, spaced at equal intervals. In practice this assembly takes little time. Slides can be prepared in advance of requirements and stored in sterile containers.

Tissue culture cells of the required type, dispensed in growth medium, are then added to each ring. Each requires approximately 0·5 ml. and the inoculum should contain 50,000–70,000 cells. The microslides, within their containers, are placed in a humidified incubator at 37°C. containing a flow of 5% CO_2 in air.

Monolayers form in the rings within 24 hours. Removal of growth medium and washing, before virus inoculation, are easily performed within the rings by a fine-bore glass pipette or large hypodermic needle attached to gentle water suction. Virus inoculation, post washing, and incubation in maintenance medium, all take place without disturbing the cultures.

For immunofluorescent identification of unknown viruses a comparatively short incubation time (four to six hours) is allowed before the medium is removed and the culture washed in buffered saline (*p*H 7·2). At this stage the rings are snapped off with forceps and dropped into hypochlorite solution. They may be washed and re-sterilized, but the cost is sufficiently low to render them disposable. As each ring is snapped off it leaves a fine circular outline of its base in the paraffin wax-petroleum jelly mixture which serves to delineate the area containing the cell culture for immunofluorescent staining.

Each slide is quickly dried in air, within an inoculation hood, and fixed in cold acetone at 4°C. for 10 minutes before staining by the immunofluorescent technique.

COMMENT

By the method described, the tissue culture worker can prepare up to six identical culture 'spots' on one microslide. The glass rings allow easy control and manipulation of the cultures for washing, changing medium, and adding virus. Each ring prevents spreading of its contents and, by the use of this technique, virus titration covering up to six dilution steps can be made on one microslide.

The great importance of this method in immunofluorescent work lies in the fact that the worker who has to stain the cultures after virus inoculation has a ring of paraffin wax-petroleum jelly within which to confine his fluorescent antibody reagents. After staining, these residual rings, which are not in themselves fluorescent in blue light, facilitate the rapid examination of the six replicate culture spots by allowing rapid interchange laterally from one spot to the other. This is important for easy reference to control cultures, stained with normal rabbit serum in the middle layer of the fluorescent

[1]The fluorescence-free glass microslides used in this investigation were obtained from Messrs. McCulloch Bros. & Wilson, 38 West Princes Street, Glasgow, C.4.

sandwich, in the definition of criteria for judging positivity for a new isolate.

SUMMARY

A new technique is described which allows rapid and easy preparation of replicate tissue culture 'spots' on glass microslides. These can be of any type of tissue culture, contained in glass rings stuck to the microslide with paraffin wax-petroleum jelly mixture. The rings allow easy manipulation of the tissue culture and the presence of up to six replicate spots on a microslide greatly facilitates the identification of unknown virus isolates from tissue culture by the immunofluorescent technique. Rapid and constant reference can be made to control preparations on the same microslide by adjusting only the lateral stage movement controls of the fluorescence microscope.

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Improved optical equipment for immunofluorescence studies[1]

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Many kinds of optical equipment for immunofluorescence are commercially available. Some are very expensive and, as far as we are aware, most if not all of them require a dark-room for efficient operation. Moreover, when viewing fluorescent structures as small as single bacteria- or virus-infected cells, the use of a binocular head on the microscope reduces the brilliance of the image to an unacceptable extent.

In view of these limitations we explored the possibility of designing and building equipment with a more intense source of radiation which should be compact and relatively inexpensive.

Although we considered other types of lamp, a very small mercury vapour arc of high intrinsic brilliance within a quartz envelope seemed to be the most promising. The lamp finally chosen was the HBO100 made by Siemens/Osram, or its near equivalent the PEK110 without starting electrode, manufactured by PEK Labs Inc., California, U.S.A. The main features of this last, taken from the maker's specifications, are as follows:

Lamp operating voltage DC (volts)	20 ± 4
Lamp rated power—DC operation—(watts)	100
Arc size (in.)	0·012 × 0·012 (0·3 × 0·3 mm.)
Average brightness (candlepower/cm.²)	140,000
Average life (hours)	100
Operating position	Horizontal or vertical
Cooling	Convection

[1]All enquiries regarding purchase of this equipment should be addressed to Messrs. Technical and Research Processes Ltd, 5 Buck Street, London, N.W.1, from whom further details including price are available.

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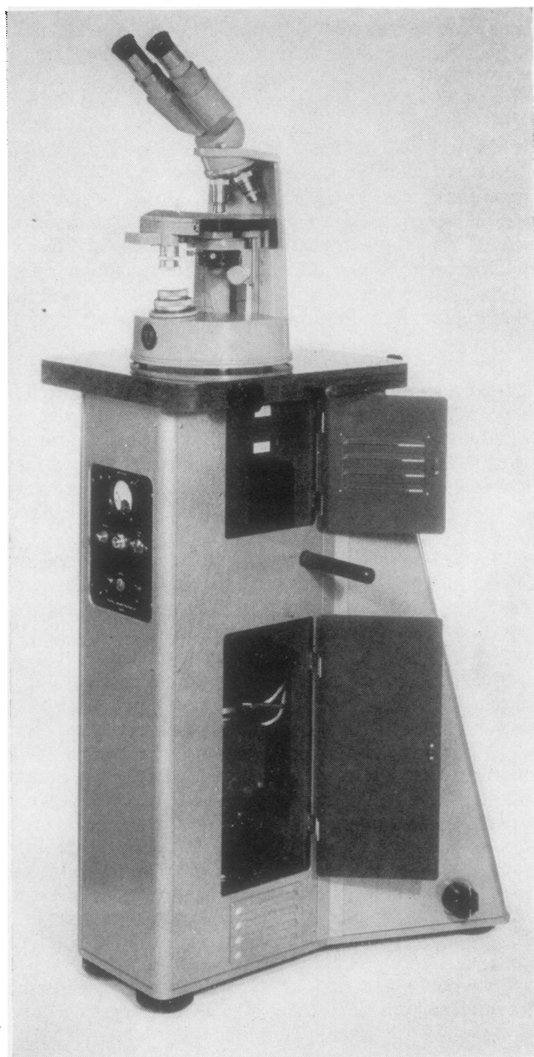

FIG. 1. *The equipment*