Automation of APAAP immunocytochemical technique

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SUMMARY A tissue processing instrument (the Histokinette) was modified by the addition of an electronic timing device which allows an immunocytochemical staining technique (the APAAP method) to be performed as a semiautomated procedure. After incubation with primary monoclonal antibodies (applied by hand) slides (up to 72 in a batch) are placed in racks and cycled through tanks of reagents, comprising anti-mouse Ig followed by APAAP complexes with intervening timed draining and washing stages. This semiautomated process gave consistent staining results and offered considerable savings in time compared with conventional methods. The same reagent baths were used over four months on an almost daily basis without deterioration in staining intensity, and consequently the calculated overall cost of the staining procedure was less than if the reagents had been applied by hand and then discarded. The machine is now into its eleventh month of operation; the reagents have been changed twice.

It is suggested that this approach, because of savings in time and increased consistency, may be an attractive technique for the routine immunocytochemical staining of slides, and that the nature of the APAAP method is particularly suitable for automation as the necessary reagents can be produced at low cost.

The APAAP immunoalkaline phosphatase technique¹ (table 1) has been widely used for staining histological and haematological specimens; the advantages of the method have been reviewed elsewhere.² APAAP staining, however, entails considerable technical time, and results depend, at least in part, on the skill and care of the person performing the staining. There would therefore be considerable benefits in a technique which automated at least part of the procedure. The manual technique is also inherently wasteful in that each of the reagents (usually in a volume of 50–100 μ l) is pipetted directly on to the slides and then washed away in buffer at the end of each incubation period. As the anti-mouse Ig and APAAP are applied at saturating concentration and are not exhausted after a single incubation, there is unnecessary wastage, which would be avoided if the reagents were reused.

For these reasons we attempted to develop an automated procedure for performing the APAAP procedure based on incubation of slides in tanks of reagents which can be used repeatedly over a period of time. Incubation with primary antibodies is the most difficult step in the procedure to automate as it is common practice to use several different reagents in a staining run, and primary antibodies are often only

Table 1 *Incubation stages in the APAAP process*

1	Incubation with primary monoclonal antibody
2	Incubation with rabbit anti-mouse immunoglobulin (rabbit anti-mouse Ig), 30 minutes
3	Incubation with immune complexes of alkaline phosphatase and anti-alkaline phosphatase (APAAP), 30 minutes
4	Optional repeat incubation with rabbit anti-mouse Ig, 10 minutes*
5	Optional repeat incubation APAAP, 10 minutes*
6	Development with a chromogenic substrate
7	Counterstaining and mounting

Slides are washed in buffer between each of the stages.

*The repetition of stages 4 and 5 enhances the intensity of the final staining reaction but further lengthens the staining procedure and so increases the possibility of technical variation.

available in very restricted amounts. We have therefore not attempted to automate this step. The final stage of the procedure (incubation in substrate) also does not lend itself to automation, given that it would entail the expense of making up a fresh tank of substrate for each staining run. We therefore concentrated on automating the middle phases of the APAAP technique, leaving the initial incubation and the final development in substrate to be performed by hand.

To simplify the construction of the staining machine and to reduce costs we based our machine on equipment which already exists in many hospitals. This approach contrasts with another published descrip-

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tion of automated immunocytochemical staining³ which uses a custom built machine.

Material and methods

TISSUE PROCESSING MACHINE

A Histokinette type E7326 (British American Optical Corporation), which had previously been used for a number of years in the histology department for tissue processing and impregnation in paraffin wax, was used (fig 1). This machine has 12 "stations" for sitting baths of reagents around the circumference of a circular platen and is equipped with heating elements to melt the paraffin wax contained in the later baths. A disc shaped roof structure has 12 points for attachment of specimen holders (or lids to cover unused baths). There is a motor driven mechanism whereby the roof is lifted, rotated one step, and then allowed to descend, transferring the specimens from one reagent bath to the next.

When the specimen is in a reagent bath a small motor moves the roof structure up and down gently by 0.8 cm, the time period of this wafting movement being four seconds.

The timing cycle of the machine is regulated by an electric clock. The time spent at each station is determined by cutting segments at appropriate intervals in the perimeter of a plastic timing disc. The shortest dwell time obtainable in any reagent bath is 20 minutes (with the variation in the time delay, expressed as a percentage of the intended time, increasing as the time delay reduces). A new timing disc is needed whenever variations are made to the times.

Modifications to tissue processing machine

The heaters were not needed and were removed and

the clock motor was disconnected electrically. The clock mechanism controlled a switch which activated the changeover mechanism. This switch was also disconnected electrically and replaced by a relay which was driven by an electronic timer.

The electronic timer: The timer was specially designed for the project and has the following features:

- 1 Twelve independently variable delays can be programmed, one for each station.
- 2 A six position switch allows the times spent at the rabbit anti-mouse Ig and APAAP stations to be varied between five and 30 minutes in five minute steps.
- 3 The timer is reset using two push button switches which need to be operated simultaneously. This is performed when the slides are loaded on to the machine at position 1. The need to activate both switches together reduces the chance of inadvertent resetting of the timer.
- 4 An alarm is sounded when the cycle is complete.

A detailed description of the timer is given in the Appendix.

Slide carriers: These were modified versions of carriers available in two sizes (Solmedia R1/3 & R1/2), enabling 36 or 24 slides to be incubated in the reagent baths. To enable suspension from the roof structure

Fig 1 Histokinette converted to perform APAAP staining: note reagent baths, specimen holder, and timer, attached to the machine by a cable.

Fig 2 Slide holder: note rubber suspension and filling in of internal spaces with silicone rubber sealant.

two 30 cm lengths of silicone rubber tubing (Altec, Alton) of 4 mm outer dimension and 2 mm inner dimension were pushed through two holes drilled in the specimen carriers of the original machine. The four ends of the tubing were slit and pushed round the vertical rods at the corner of the Solmedia holders (fig 2). The length and position of the rubber tubes were adjusted to give the side holders a tilt of about 15° in the direction of the slides' long axes and the tubing secured in place with PVC tape. The tilt reduces the amount of the reagents that remains as droplets on the underside of the slides when they are lifted out of the reagent baths. The non-rigid rubber suspension assists the gentle carriage of the slides and also means that the exact positioning of the baths below the suspension points is not a critical factor as the slide holders tend to self-centre during descent into the reagent baths. This allows slide holders to be used which fit snugly into the reagent baths and it maximises the number of slides that can be stained in a vessel of given capacity. The rubber tubes flex when the slides reach the level of the platen which allows the holders to sit horizontally in the reagent baths.

The slides are put into the holders back to back. Care is taken to place the slides in such a way that the holder is balanced. It is disadvantageous to put slides in zig zag fashion as, after immersion, a meniscus forms at the V junctions between the slides causing an increase in carry-over from one bath to the next. To reduce the carry-over further we minimised the possibility of capillary action within the slide holders themselves by filling the spaces between the corrugations and the holder side (fig 2) with silicone rubber aquarium sealant (Aquacare). When the slides are in the holders, the proximity of the slides to each other prevents them from drying out during the transfer from one reagent bath to the next.

Vessels and reagents: Flexible plastic reagent vessels (Stewart, No 281) were used. The volume of rabbit anti-mouse Ig, APAAP, or buffer needed to cover the slides is 400 ml. When not in use the rabbit anti-mouse Ig, APAAP, and the drain pots are stored in their plastic vessels at 4°C. The buffer vessels are left covered at room temperature.

The buffer used is the same as that used for the manual method (Tris-hydrochloride 0.05 M in isotonic saline, pH 7.6) except that 0.02% sodium azide is added to inhibit bacterial growth. Rabbit anti-mouse Ig (Dakopatts, No Z109) was prepared by dilution 1 in 50 in Tris-buffered saline (TBS) with the addition of 40 ml of normal human serum. The APAAP complexes were prepared from 400 ml of monoclonal mouse anti-alkaline phosphatase hybridoma culture supernatant by the addition of 3.2 g of calf intestinal alkaline phosphatase (Sigma P3877) and 0.02% sodium azide. To further inhibit

bacterial growth gentamicin (20 µg ml⁻¹) was subsequently added to the rabbit anti-mouse Ig and APAAP.

TIMER OPTIMISATION

The times shown in table 2 were found to be effective. The time for the turning mechanism to operate is about one minute so the total cycle time is 20 minutes plus twice the incubation time selected for stages 9 and 3. This works out at between 30 and 80 minutes, depending on the length of the delay chosen for the rabbit anti-mouse Ig and APAAP.

The time periods of the timer described above are the same for steps 1–6 as for steps 7–12. This symmetry means that it is possible to attach another slide carrier to the machine when the first slide holder is at station 6. This ability to double up the holders means that a maximum of 72 slides can be on the machine at a time. With a total cycle time of 70 minutes the machine could stain several hundred slides in a day.

REAGENT CYCLE

Each complete cycle on the machine has 12 stages (table 2) and carries slides once through the anti-mouse and APAAP (stages 2 and 3, or 4 and 5 of table 1). Several methods are used to maximise reagent life, prevent carry-over, and prevent the mixing of rabbit anti-mouse Ig and APAAP.

(a) The rabbit anti-mouse Ig and APAAP which remains on the slides immediately after they have been lifted out of the two reagent baths can be partially recovered by a drain step in which the slide holders touch down into a vessel containing 30 ml of buffer. This vessel is raised 3.2 cm above the platen and the drops of rabbit anti-mouse Ig or APAAP on the bottom of the slides drain into the buffer, the presence of buffer serving to overcome the surface tension effect that would prevent the drops from wetting a dry plastic surface. The contents of the drain vessel are emptied into the preceding one at the end of the week,

Table 2 *Details of the automated staining cycle*

Station	Reagent	Time
1	Buffer	2 minutes
2	Buffer	16 seconds
3	Rabbit anti-mouse Ig	5–30 minutes in steps of 5 minutes†
4	Drain	16 seconds
5	Blot	16 seconds
6	Buffer	2 minutes
7	Buffer	2 minutes
8	Blot	16 seconds
9	APAAP	5–30 minutes in steps of 5 minutes†
10	Drain	16 seconds
11	Blot	16 seconds
12	Buffer	2 minutes

†This time is selected with a six position switch. The alarm sounds as the slides are lifted from buffer at the end of the wash in stage 12. The slides come to rest in the buffer at station 1 and move no further unless the timer is reset.

thereby recovering the rabbit anti-mouse Ig and APAAP and also making up for loss of volume due to evaporation.

(b) Slides are blotted dry by allowing the slide holders to touch down on to absorbent paper covering a box 6 cm high. This dries the bottom of the slides where drops of reagent or buffer tend to accumulate.

(c) There are two wash stages between the rabbit anti-mouse Ig and the APAAP.

At the end of the week buffers are changed and the contents of the drain pots added to the preceding reagent bath. If many slides are being processed the buffers are changed more frequently.

RUNNING PROCEDURE

Before starting a run the position of the reagent baths is checked and dry paper tissues put in place for the blot stages. The slide holder and slides are then attached to the machine at position 1 with the slides in the buffer. The appropriate incubation delay is selected with the six position switch. The timer is reset, starting the cycle, and the machine left unattended till the end of step 12 in the cycle. If a repeat step is not required the slides are then removed. If repeats are required the timer is reset, without the need to move the slides, and the incubation delay for the next cycle is selected. At the end of the repeat cycle the slides are ready for development.

Results

Semiautomated APAAP staining by the technique described in this paper has now been in use in the authors' laboratory for 11 months. The intensity of staining has been equal to, or on occasions stronger than, the manual method. In some cases the increased intensity of staining with the machine has allowed a more dilute primary antibody to be used than is needed for manual staining.

After the first month of operation the rabbit anti-mouse Ig and APAAP became contaminated with bacteria although the staining quality did not deteriorate. This was detected by slight turbidity of the reagents and an offensive smell, and by a mixed growth of facultative anaerobes on culture. Most of the organisms were sensitive to gentamicin and the reagents were therefore filtered and this antibiotic then added.

During the first four months of use the initial batch of rabbit anti-mouse Ig and APAAP was not changed but subsequently the intensity of staining began to decline slightly. The rabbit anti-mouse and APAAP on the machine were replaced with fresh reagents and the machine continues to work satisfactorily with a third batch of reagents. The reagents which had been removed from the machine were not completely

exhausted and have proved adequate for use in the manual method.

The machine has stained an average of 20 slides a day, mostly with repeat cycles. About half of the slides have been multiwell slides each of which carry four tissue sections. With the manual method each slide takes an average of 200 μ l of reagent at each step—that is 400 μ l of each when a repeat cycle is preferred. Therefore staining of 1000 slides by the manual method would consume about 400 ml of each reagent. This represents the "break-even point", in that any staining which can be performed with the reagents by the automated procedure beyond this number would be more economical than the manual method. In the authors' laboratory this point was reached after about 10 weeks and the staining quality was not diminished until four months of use. When using multiwell slides and cryostat sections (for example when screening for monoclonal antibodies after a hybridoma fusion) the machine could stain 200 multiwell slides a day and the break-even point would be reached in a matter of days.

Discussion

Rotary tissue processors are available but unused in many hospital pathology laboratories, having been replaced by newer microprocessor controlled instruments. By the addition of a simple but flexible electronic timing device the Histokinette can be modified to cycle batches of slides through a sequence of immunocytochemical reagents. The advantages of automatically cycling slide holders through tanks of reagents compared with a manual batch approach⁴ are reduced technician workload and the assurance of accurate timing. These benefits are greatest when repeat stages are needed. The machine also has the facility to oscillate gently the holders in the tanks which may speed up the attainment of equilibrium between slides and the reagents. With 12 stages on the machine it is easy to incorporate a method of recovering the drops of reagents which adhere to the slides; reusing these reagents keeps down the costs.

The procedure described in this paper could be used to automate any of the techniques currently used for immunocytochemical staining. The APAAP procedure, however, lends itself particularly well to automation. One reason is that the two major reagents (anti-mouse Ig and APAAP complexes) are stable and can be used repeatedly over long periods. More importantly, each of these reagents lends itself better to large scale production at low cost than do the reagents used in other immunocytochemical procedures. The reasons for this are considered below: *Production of anti-mouse Ig*: The production of this reagent in essence requires little more than the immunisation of an animal with mouse Ig (which need not be

highly purified—see below) and harvesting of the serum. No chemical modification of the anti-mouse Ig is needed (as is required when an enzyme linked conjugate is prepared), and indeed it is not even necessary to isolate an Ig fraction, as the serum should work equally well in a crude unfractionated state. Furthermore, the antiserum can contain contaminating antibodies against antigens other than mouse Ig without affecting the specificity or quality of the APAAP staining reaction. This is due to the role which the anti-mouse Ig has in the APAAP reaction (fig 3), in which it actively binds the mouse immunoglobulin in APAAP complexes in the succeeding layer. This “bridging” role is quite different from the function of anti-mouse Ig in an “indirect” procedure (such as a three stage immunoperoxidase technique) in which any contaminating antibody in the second layer of the sandwich which binds to the specimen will give rise to non-specific labelling (fig 4). In contrast, any such antibodies present in the anti-mouse Ig used in the APAAP procedure cannot cause non-specific staining as they lack anti-mouse Ig reactivity and hence cannot bind the APAAP complexes (fig 3).

The only risk of non-specific staining at this stage of the APAAP process arises from cross reactivity of the rabbit anti-mouse Ig with human immunoglobulin, which might cause the APAAP complexes to be bound to human immunoglobulin in the specimen (for example, to plasma cell cytoplasm). Anti-human Ig activity, however, can be eliminated either by absorption with insolubilised human Ig, or more easily, by the addition of human serum (as in this study).

Production of APAAP complexes: This reagent is clearly a crucial element in the APAAP procedure as it contains the enzyme which gives the final colour reaction, but it is a simpler reagent to prepare than the enzyme conjugates used in indirect immunoenzyme procedures. In such methods the enzyme has to be covalently conjugated (for example, by using a bifunctional reagent such as glutaraldehyde) to antibody. This step is subject to batch to batch variation and hence requires careful quality control. Furthermore, the enzyme used for covalent coupling methods needs to be of high purity, and it is necessary to check at the end of the conjugation process for products liable to cause staining that is non-specific or of reduced intensity (for example, high molecular weight complexes, unconjugated antibody, etc.).

The production of APAAP complexes, in contrast, is free of these constraints as the complexes are prepared simply by adding calf intestinal alkaline phosphatase to a solution containing monoclonal antibody specific for the enzyme (fig 5). In the authors' laboratory the latter solution is in the form of tissue culture supernatant, directly as harvested from the hybridoma cell line. The alkaline phosphatase

preparation can be of low purity as the monoclonal mouse antibody will react specifically with the enzyme, and impurities should not prevent the generation of APAAP complexes (fig 5), or interfere with the function of these complexes.

As noted above, the automated method uses

Fig 3 Diagrammatic representation of the APAAP technique showing, on the left, specific staining of antigen and, on the right, binding of contaminating second layer antibodies, which causes no unwanted non-specific staining.

Fig 4 Diagrammatic representation of three stage peroxidase technique showing specific staining on the left, and, on the right, the background staining resulting from impurities in the second layer reagent.

Preparation of APAAP Complexes from unpurified reagents

Fig 5 Diagrammatic representation of the formation of APAAP complexes: note that only a mixing step is required and that, despite impurities in the starting reagents, the only molecules which contribute to complex formation are alkaline phosphatase and anti-alkaline phosphatase.

reagents more efficiently than manual techniques or another automated method³ as the reagents are not discarded. Furthermore, the stronger staining reaction achieved in some cases has enabled a higher dilution of primary antibody to be used, while semiautomated staining also carries a cost benefit in terms of reduction in operator time. At today's prices, however, (Dakopatts catalogue), the reagents needed to fill the 400 ml baths would cost about £100 for the rabbit anti-mouse Ig and £840 for the APAAP, and this initial outlay is likely to limit the use of the procedure on a wide scale. Perhaps manufacturers (in view of the inherent simplicity of preparing anti-mouse Ig and APAAP, as discussed above) will consider reducing their prices, possibly by producing these reagents in bulk form specifically intended for the semiautomated procedure.

When the use of a machine to cycle slides through baths of reagents was first considered we foresaw potential problems arising from the carry-over of reagents due to insufficient washing. In consequence, the APAAP complexes would increasingly be con-

taminated with anti-mouse Ig and vice versa, thereby reducing the activity of these two reagents. In studies using changes in weight of the absorbent tissues and dyes as markers of carry-over, however, we established that the sequence of steps described in this paper (table 2) avoids this problem. Of these, the draining step immediately after the anti-mouse Ig and the APAAP baths is important as it removes most of the carried-over reagent and allows it to be returned to the appropriate bath. In the subsequent blotting stage any residual drops are removed. We have not attempted to measure accurately the degree to which carry-over occurs but it cannot be substantial given the consistently strong staining which is achieved after several months of operation.

Despite being partly made from discarded equipment, the machine described above has proved reliable. The electronic timer represents the only major modification to the machine, and it would be easy in a few minutes to return the machine to its native mode, based on the original timer, for use as a backup tissue processor if required.

The technique described in this paper has proved suitable for many types of specimen, such as cryostat and paraffin wax sections, cytospins, multispots, blood smears and bone marrow aspirates. Unlike other automated immunostaining equipment,³ the machine accepts all standard microscope slides and is unaffected by variations in the physical dimensions or the position on the slide of the area to be stained. It has the potential to be widely used in hospital laboratories and should help to transform immunocytochemical staining from a specialised research procedure into a routine technique requiring minimal operator time.

Appendix

THE ELECTRONIC TIMER

The timer is based on integrated circuit R-C oscillator; the speed of oscillation is controlled by an external resistor and capacitor. A digital frequency divider (dividing by 64) is used subsequently so that the primary oscillator's speed can be sufficiently fast for it to be accurate when timing 30 minute delays. After 64 input pulses the frequency divider gives an output pulse to the input of a counter. The intervals between these lower frequency pulses dictate the time period for which the slides remain at any stage in the cycle. The counter's outputs are a series of 13 points which are normally at low voltage but which are each taken to high voltage (+12 V DC) in turn for one period before returning to 0 volts. The first 12 of these points correspond to each of the 12 stations and the last one, representing the end of the run, drives an audible piezoelectric alarm. Each of the first 12 points when at high voltage drives: (a) a light to indicate the state of

the timer circuit and to show the position to which the specimen holder should have moved; (b) a resistor which provides the current source for the oscillator. The speed of the primary oscillator is therefore controlled by the value of this resistor. This enables independent control of each of the 12 incubation steps. A change in the delay at any given station is achieved by changing a single resistor. (If it is necessary to vary the delays of *all* the incubations, while retaining the relative proportions of each, it is possible to substitute the capacitor of the R-C oscillator for one of an appropriate value.) The thirteenth point is not connected via a resistor to the oscillator and therefore the oscillator stops and the alarm sounds. During this time there is no possibility of the slides progressing further than intended as the oscillator is stopped.

At the end of each of the 12 delays the circuit drives a relay to activate the changeover mechanism of the Histokinette, thus moving the slide holder on to the next station. This changeover takes about 50 seconds. To ensure accurate short incubations the oscillator is inhibited while the relay is activated and slides are in the air. The next timing period starts only when the slides have reached the next bath.

The timer circuit, which uses six integrated circuits plus other components such as resistors, capacitors and diodes, is constructed on Vero board and housed

in a splashproof box with a clear lid. It is attached to the Histokinette, from which it derives its power, by a flexible cable. All mains voltage components are retained within the earthed Histokinette chassis thus ensuring electrical safety.

All the components, including a regulated 12 volt power supply, can be purchased for less than £60. The circuit construction is well within the capabilities of the amateur electronics enthusiast. A circuit diagram for the timer is available from the authors.

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